BACTERIOLOGICAL ANALYSIS WITH ANTIBIOTIC RESISTANCE PATTERN OF BACTERIA ISOLATED FROM FRESH FRUIT JUICES OF DINAJPUR CITY, BANGLADESH

A THESIS

BY

MOHANANDA SARKAR

REGISTRATION NO. 1605125

SEMESTER: JANUARY–JUNE, 2017

MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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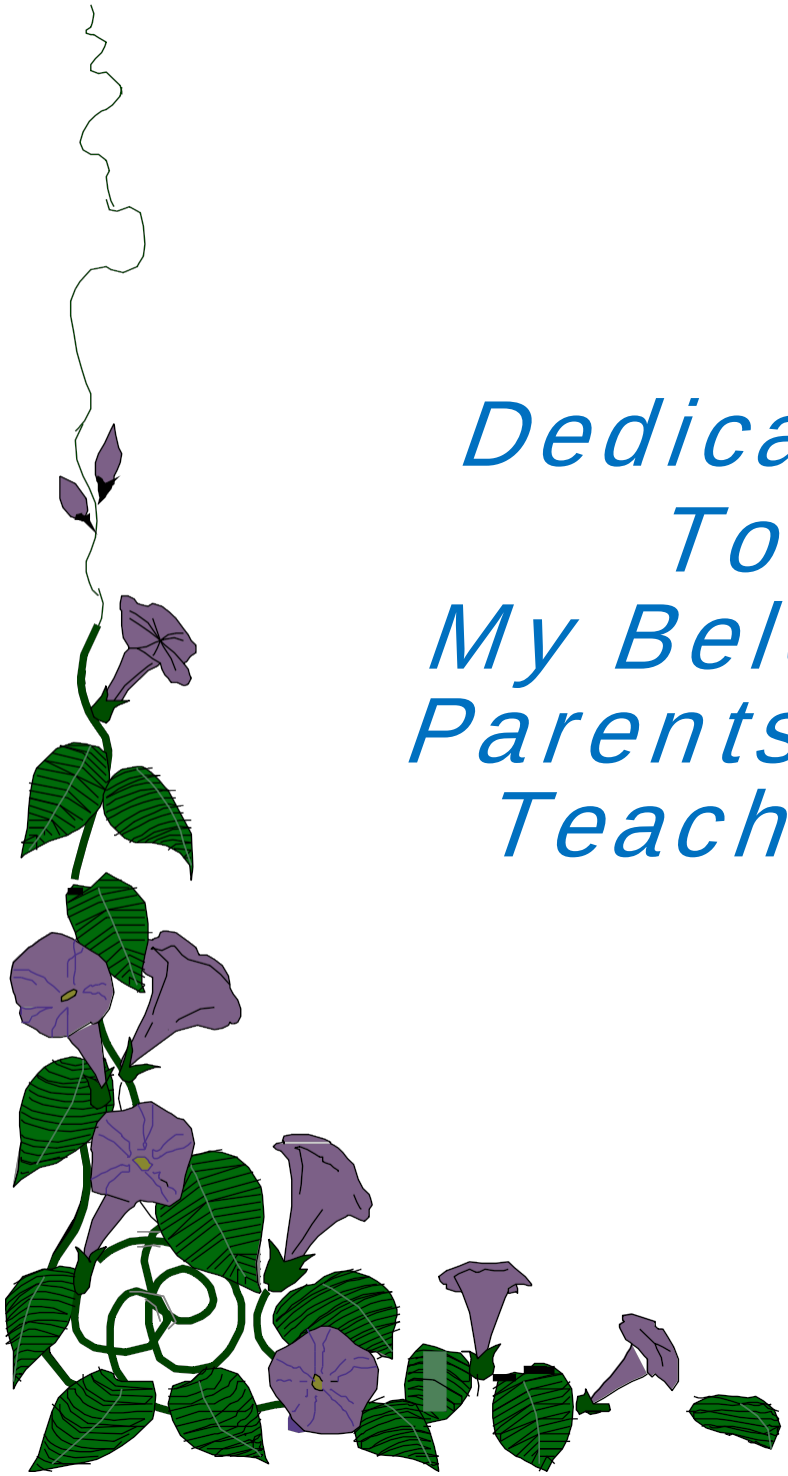

Dedicated
To
My Beloved
Parents and
Teachers

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ABSTRACT

Freshly prepared fruit juices sold by local market vendors in Dinajpur city were analyzed for the bacteriological quality. Forty (40) fresh fruit juices were collected from different areas around Dinajpur city. Standard plate count techniques were followed to assess Total viable bacterial count (TVC), Total coliform count (TCC), and Total Staphylococcal count (TSC). The total viable bacterial count ranged from 1.8×10^4 to 5.6×10^7cfu/ml. Total coliform count and Total Staphylococcal count of juice samples were ranged from 1.36×10^3 to 5.76×10^6 cfu/ml and 0 to 5.85×10^6 cfu/ml respectively. The juice samples were also found to be contaminated with *Escherichia coli, Staphylococcus* spp., *Salmonella* spp. & *Klebsiella* spp. Out of 40 freshly prepared fruit juice samples collected, 38 samples (95%) showed the presence of *E. coli*. The percentage of *Staphylococcus* spp., *Salmonella* spp. & *Klebsiella* spp. of the tested samples were 75%, 12.5%, and 10% respectively. So the fruit juice samples were unsafe for drinking. Antibiotic resistance pattern of the isolated bacteria revealed that, among the four isolated bacteria *E. coli, Salmonella* spp., *Klebsiell*a spp. were 100% resistant to Ampicillin but *Staphylococcus* spp. were 87% resistant to Ampicillin. Isolated *Klebsiella* spp. was found to be 75% resistant to Amoxicillin and *Salmonella* spp. were 20% resistant to Vancomycin. Such drug resistance properties may render these pathogens cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics. It was concluded that due to unhygienic fruit handling in the unsanitary environmental conditions

the juices become contaminated with bacteria which are harmful for public health.

Key words: Fruit juice, TVC, TCC, TSC, Antibiotic Resistance pattern.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	:	Negative
%	:	Percentage
/	:	Per
<	:	Less than
>	:	Greater than
+	:	Positive
µg	:	Microgram
µl	:	Micro liter
^{0}C	:	Degree of Celsius
CFU	:	Colony forming units
D_x	:	Dextrose
E. coli	:	*Escherichia coli*
e.g	:	Example
EMB	:	Eosin Methylene Blue
et al.	:	Associated
Etc	:	Etcetera

FAO	:	Food and Agricultural Organization
Fig.	:	Figure
Gm	:	Grams
H_2S	:	Hydrogen sulfide
Hrs	:	Hours
HSTU	:	Hajee Mohammad Danesh Science and Technology University
Lb	:	Pound
Kg	:	Kilogram
KOH	:	Potassium hydroxide
L	:	Lactose
MC	:	MacConkey Agar
Mg	:	Milligram
Min	:	Minutes
MI	:	Milliliter
MIU	:	Motility Indole Urease
Ml	:	Milliliter

LIST OF ABBREVIATIONS AND SYMBOLS (Contd.)

ML	:	Maltose
Mm	:	Millimeter
MN	:	Mannitol
MR	:	Methyl Red
MSA	:	Mannitol Salt Agar
N	:	Number
NA	:	Nutrient agar
NB	:	Nutrient broth
ND	:	Not done
-	:	Negative
No.	:	Number
PBS	:	Phosphate Buffer Saline
R	:	Resistant
S	:	Sucrose

S	:	Sensitive
Sec	:	Second
SL.	:	Serial
spp.	:	Species
Sq	:	Square
SSA	:	Salmonella Shigella Agar
TCC	:	Total coliform count
TSC	:	Total Staphylococcal count
TSI	:	Triple sugar iron
TVC	:	Total viable count
VP	:	Voges Proskaur
v/v	:	Volume by volume
w/v	:	Weight by volume

CHAPTER 1

INTRODUCTION

Fruit juices are nutritious drinks which offer great taste and health benefits (Suaad and Eman, 2008). Fruit juices are becoming an important part of the modern diet in many communities. They are nutritious beverages and can play a significant role in a healthy diet because they offer good taste and a variety of nutrients found naturally in fruits (Tasnim et al., 2010). Nowadays, the demand for freshly squeezed fruit juices in comparison to bottled or canned juices has increased as the consumer prefers unpasteurized juices because of the fresh flavor and the absence of preservatives. Fresh fruit juices have no artificial color and sweetness is natural that is why they are preferred over bottled or canned juices (Melbourne, 2005; Addo et al., 2008). Freshly squeezed juices are simply prepared by extracting the liquid and pulp of mature fruit usually by mechanical means or blenders. Improperly prepared fresh fruits and vegetable juices are recognized as an emerging cause of food-borne illnesses (Sandeep et al., 2004).

There are many reports of food borne diseases due to the consumption of fruit juice at several places around the world (Mosupye and Holy, 2000; Muinde and Kuria, 2005). The major ingredients of the juice such as water, sugar, natural fruit pulp etc may also carry some microbial contaminants. Food-borne illness is commonly caused by certain bacteria or their toxins, which are poisonous proteins produced by these bacteria (Bryan, F. L. 1977). Several factors can act as source of microbial contamination of the fruit juices such as use of unhygienic water for dilution, dressing with ice, prolonged preservation without refrigeration, unhygienic surroundings often with swarming houseflies and fruit flies and airborne dust (Tasnim et al., 2010; Babalola et al., 2011; Odu and Adeniji, 2013). However, in the absence of good manufacturing process nutritionally rich components of fruit juices makes the product, acts as a good medium for microbial growth and vehicle for food borne pathogens

(Ketema et al., 2001). The most common food borne pathogenic bacteria are *Bacillus cereus*, *Clostridium botulinum, Escherichia coli, Shigella* spp., *Salmonella* spp., *Vibrio parahaemolyticus, Staphylococcus aureus, Campylobacter jejuni, Streptococcus pyogenes, Mycobacterium bovis*, *Listeria monocytogenes* etc (Prescott, L. M et al., 2002). Various authors have also reported the presence of pathogens, namely, *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Staphylococcus aureus* (Sandeep et al., 2004; Rashed et al., 2013). The antimicrobial resistance of bacteria isolated from food and other sources, against commonly used antibiotics has increased from time to time (Vicas and Singh, 2010). Not only their presence, but also their resistance to the commonly used antibiotics has become a concern for consumers. Some reports have revealed that antibiotic resistance levels are becoming elevated among food-borne pathogens such as *Salmonella* and *Shigella* (Mache, 2002). Although, it is difficult to prove a direct role of drug resistance in bacteria contaminating food items with increased clinical cases of resistant infections, the presence of such bacteria in food items could play a role in the spread of antimicrobial resistance among food-borne pathogens (Farzana et al., 2009). The incidence of resistant bacteria in foodstuff is a worldwide phenomenon. It is a major public health threat (Khan and Malik, 2001) as these organisms have been isolated from wide range of foodstuffs consumed by human. In the developed countries, the quality of fruit juices is strictly being maintained under several laws and regulations, whereas in many developing countries including Bangladesh, the manufacturers are not much concerned about the safety and hygiene of fruit juices because of lack of enforcement of the law. Thus the transmission of certain human diseases through juice and other drinks becomes a serious problem (Tasnim et al., 2010).

In Dinajpur city there is a great demand of fresh fruit juices as the climate remains very hot for most part of the year. While most restaurants and cafe serve juices in apparently hygienic conditions, unfortunately in roadside shops, recreational areas (parks), the microbiological quality of the supplied juices remains questionable. In

view of the threat posed by the bacterial pathogens in fresh fruit juices of such local market and street vended juices, the present work was undertaken to assess the bacteriological quality with antibiotic resistant pattern of isolated bacteria of fresh fruit juices and their safety for human consumption in terms of bacterial pathogens.

So the objective of this study is,

i. Evaluation of bacteriological quality of fresh fruit juices collected from different areas around Dinajpur city by assessing their microbial load and the presence of pathogenic bacteria.

ii. Determination of levels of Total viable count (TVC), Total coliform count (TCC) and Total Staphylococcal count (TSC) in the fresh fruit juice samples.

iii. Test the antibiotic resistance pattern of the isolated bacteria.

CHAPTER 2

REVIEW OF LITERATURES

Addisu Desalegnet *et al.* (2016) conducted a study for the isolation and identification of bacteria from fresh juice prepared in cafeterias and restaurants. Thirty Samples of Avocado and Mango locally prepared fruit juices were collected randomly from different restaurants and cafeterias of Axum town. Microscopic investigation for Gram reaction and morphological features of suspected colony was determined using standard method of Gram‘s staining. Results showed that, in Mango and Avocado sample, sample 10-1 was most contaminated with a count of 150 and 120 coliforms per 100 ml of the juice sample, respectively. The second highest contamination was seen in juice sample 10-2 with a count of 100 and 100 coliforms per 100 ml of Mango and Avocado.

Kaniz Fatema *et al.* (2016) conducted a bacteriological study of handmade juice in street of Dhaka city. For this total viable bacterial count (TVBC) isolation, purification, Gram staining, selective isolation, result interpretations were determined in Mango juice (*Mangifera indica*), Apple juice (*Malus domestica*), Orange juice (*Citrus sinensis*), Malta juice (*Helichrysum melitense*) and Lacchi. In such investigation highest TVBC ($1.4x10^6$) and ($1.2x10^6$) was observed in Mango juice and Alovera juice which is form Khilkhet (street) and Sadarghat (street) and the lowest TVBC ($9.0x10^5$) was observed in Malta juice which is collect form banani($1.2x10^6$) and TVBC ($9.0x10^5$) was observed in Papaya which is collect form Banani. *Enterobacter aerogenes* was present in Mango juice sample, *Pseudomonas aeroginosa* was present in Apple juice sample*, Salmonella typhimurium* was present in Malta juice sample, *Bacillus cereus* was present in Orange juice sample and *Klebshilla pneumoniae* was present in Lacchi sample.

K. Sahithi Reddy *et al.* (2016) determined the pH and specific microorganisms in freshly squeezed street vended fruit juices. Four fruit juices i.e., Grapes, Sweet Lime, Pineapple and Sapota were chosen for

the study. Juices were collected in summer season in months between April and June 2013. Ten samples of 50 ml each fruit juice was collected in sterile bottles from various street vendors of Dilshuknagar area of Hyderabad city. All juices showed bacterial contamination except one sample of grape juice. Pineapple juice samples showed the high bacterial contamination with all samples positive for fecal coliforms and *Shigella* spp. (100%). *Salmonella* spp. was detected only in one sample of Sapota juice (10%). Significant difference among fruit juices for prevalence of microorganisms was seen only for *Escherichia coli* (P = 0.03) with least count in Grape juice (20%). Freshly squeezed street vended fruit juices were contaminated with pathogenic bacteria, which significantly attributed to public health problem.

Ogodo, A.C. *et al.* (2016) assessed the microbiological quality of commercially packed fruit juices sold in South-East Nigeria. A total of forty (40) juice samples consisting of orange, apple, pineapple, lemon, and guava flavoured varieties were collected. They observed the highest total bacteria load of 4.4×10^5 cfu/ml in sample A (Orange) while the lowest in sample D of Apple variety (1.95×10^4 cfu/ml). The total coliform count ranged from no count in samples A, B, C and D to 9.8×10^1 cfu/ml in sample I (Guava juice). The staphylococcal count ranged from no count in samples E, F, I and J to 8.4×10^2 cfu/ml in sample G (lemon juice). The microorganisms isolated from the samples included *Staphylococcus aureus, Bacillu*s species, *Enterobacter* species, *Acetobacter* species, *Lactobacillus* species, *Saccharomyces cerevisiae, Aspergillus* species, *Rhizopu*s species and *Penicillium* species. Bacillus species was the most common (70%), followed by *S. aureus* (60%), *Enterobacter* spp.

Bikorimana Jean Pierre *et al.* (2015) evaluated the quality of orange fruit juices sold by street vendors alongside of roads in Chidambaram, Tamil Nadu, India. The three bacteria were identified after isolation in a specific culture medium and performing the biochemical tests, those bacteria are *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus.* Overall results demonstrated that non-hygienic quality of street vended orange fruit juices and ice used for cooling of juices suggesting

the urgent need for Government participation in developing suitable intervention measures to improve microbial quality of orange juices.

E. Simforian *et al.* (2015) conducted a study to assess the bacterial quality and establish the risk factors for contamination of raw fruit juices vended in Dar es Salaam city, Tanzania. Ninety fruit juice vendors were assessed for possible factors of microbial contamination in fruit juices. The results showed that the total plate counts (TPC) ranged between 2.32 and 8.54 (Log cfu/ml). About 72.2% of juice samples had TPC above Codex recommended maximum levels (3.7e4.7 Log cfu/ml). The prevalence of *Escherichia coli* in the juices was 80% with a range between 0.0 and 5.0 (Log MPN/ml) suggesting of direct faecal contamination or contamination from the environment.

Inderdeep Kaur *et al.* (2015) analyzed 40 Vended Street Fruit Juices samples, collected from the Allahabad City and founded that *E.coli*, *Salmonella* sp., *L. casei, L. acidophilus* were present in the samples. The contamination is mainly due to poor quality of water used for dilution, washing of utensils, contaminated ice, poor personal and domestic hygiene, peeling of fruits beforehand and shops in crowded places.

Mahbub Murshed Khan *et al.* (2015) assessed the microbiological load, possible risk factors and identity of freshly squeezed juices and sherbets and their safety for human consumption in terms of pathogens.For the study purpose papaya juice, sugarcane juice, tukmaria sherbet, lemon sherbet and wood apple sherbet were taken as samples. The study showed a high microbial load in the drinks. The range of average total viable count (microbial load) and total coliforms were 7.7×10^3 - 9×10^8 cfu/ml and 210–1100 cfu/100 ml, indicated the heavy presence of microorganisms in all the drinks analyzed in this study. The study revealed that tukmaria sherbet was most contaminated with a count of 9×10^8 cfu/ml. The least contamination was observed in lemon sherbet. A count of 1.98×10^6 cfu/ml was observed in papaya juice and 3.4×10^5 cfu/ml was in wood apple sherbet. Total coliforms were present in all samples and average count for total coliforms was high in tukmaria

sherbets than others. Various pathogenic species of bacteria such as *Proteus* sp*., Enterobacter* sp, *E. coli, Shigella* sp, *Citrobacter* sp*, Vibrio* sp, *Yersinia* sp and *Hafnia* sp were isolated from the juices and sherbets. Unhygienic water for dilution, dressing with ice, prolonged use without refrigeration, insanitary surroundings, raw materials, chemical properties, equipment, fruit flies and airborne dust are the risk factors of contamination.

Muhammad Naeem Iqbal *et al.* (2015) determined the microbial load of un-pasteurized packed fruit juices sold in Lahore city and to determine antibacterial activity of five different honey samples against isolated bacteria. All the samples were subjected to Total viable count (TVC), Staphylococcal count (SC) and Coliform count (CC). They isolated one hundred and ten strains of bacteria were isolated from various fruit juices and identified on the basis of cultural characters, morphology and biochemical characters. Mean TVCs, SCs and CCs of juices (6.80±1.91, 5.45±1.06 and 3.25±1.25 log10 CFU/ml respectively) were non-significant with standard permissible limits ($p<0.05$). Among all the fruit juices, 66.66% of samples had TVC more than 4 log10 CFU/ml, 51.66% of samples had SC more than 3 log10 CFU/ml and 46.66% of samples had CC more than 2 log10 CFU/ml.

Asha S. *et al.* (2014) evaluated the quality of juices sold by street vendors in Guntur, A.P., India. TVC (Total Viable Count), coliforms and yeast counts were analysed using standard methods like serial dilution and plate count. The results showed that TVC was highest in carrot juice followed by beetroot, pineapple, grape and mousambi juices respectively. TVC count ranged from 87-250 (x 10^5 CFU/ml) in beetroot juice; 80-271 in carrot juice; 15-150 in mousambi juice; 19-184 in grape juice and 25-217 in pineapple juice. The coliform count ranged from 8-66 (x 10^5 CFU/ml) in beetroot juice; 1-54 in carrot juice; 0-73 in mousambi juice; 1-10 in grape juice and 1-63 in pineapple juice respectively.

Bello Olorunjuwon O. *et al.* (2014) conducted an experiment for microbiological assessment of fruit juices, 120 fruit juice samples (24

each of avocado, papaya, pineapple, grape and orange) were collected. The spread plate method was used for the isolation of bacteria on appropriate selective media. Isolated bacteria were *Staphylococcus aureus, Escherichia coli, Klebsiella* sp, *Pseudomonas aeruginosa*, *Bacillus cereus, Enterobacter* sp, *Salmonella* sp, *Streptococcus* sp, *Proteus* sp and *Serratia* sp. They also found that the mean total viable count was highest in papaya juice ($6.5x10^4$ cfu/ml) and lowest in grape juice ($4.0x10^4$ cfu/ml). Yeast count was highest in orange juice ($3.5x10^4$ cfu/ml) and lowest in grape juice ($2.0x10^4$ cfu/ml). Papaya and grape juices recorded the lowest mold count ($2.7x10^4$ cfu/ml).

Chandi C. *et al.* (2014) aimed at to study the presence of food borne pathogens (Bacteria and Yeasts) in street vended fruit juices and investigated the antimicrobial activity of ten essential oils, for a potential use in food industries. Forty one samples of four different types (Orange, Grapes, Mosambi and Sugarcane) of fruit juices were collected from vendors following standard practices. They observed their sixty percent of the samples were positive for presence of coliforms. Pathogenic bacteria like *Arizona* sp., *Bacillus* sp., *Escherichia coli, Enterobacter* sp., *Enterococcus., Klebsiella pneumoniae*, *Proteus mirabilis, Pseudomonas* sp., *Salmonella typhi, S. paratyphi, Shigella* sp., *Staphylococcus* sp. and *Streptococcus faecalis* and yeasts like *Candida tropicalis, Candida glabrata* and *Candida* spp. were detected, is indicative of fecal and water borne contamination of these fruit juices.

Kamal Rai Aneja *et al.* (2014) conducted a microbiological examination of freshly prepared juices (sweet lime, orange, and carrot) by serial dilution agar plate technique. They examined total 30 juice samples for microbiological quality. They isolated twenty-five microbial species including 9 bacterial isolates, 5 yeast isolates, and 11 mould isolates were isolated from juices. Among bacteria *Bacillus cereus* and

Serratia were dominant. *Escherichia coli* and *Staphylococcus aureus* were detected in few samples.

Md. Munjur *et al.* (2014) investigated to resolve the microbiological attributes of the fruit juices collected from different areas around Jessore city. Ten fresh fruit juices and ten commercially packed fruit juices were collected. Standard plate count techniques were followed to assess total viable count (TVC), total coliform count (TCC) and total Staphylococcal count (TSC) on different culture media. Samples were found to harbor viable bacteria within the range between 10^3-10^8 cfu/ ml. 19 samples exhibited the presence of Staphylococci. Total coliforms were detected in 17 samples within the range of 10^3-10^6 cfu/ ml which were further detected as *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp.

O. K. Agwa *et al.* (2014) evaluated the microbial quality of some industrial and locally made fruit juice. A total of twenty samples of orange fruit juices were collected for analysis from different locations in Port Harcourt. The Total Heterotrophic Bacteria Count (THC) for locally made juices ranged from 1.1 – 5.0 x 10^3 CFU/ml, a THC of 1.0 x10^3 - 4.0 x 10^3 CFU/ml was recorded in industrially processed samples. Total Fungal Count (TFC) ranged from 1.0 – 7.0 x 10^2 CFU/ml, for locally produced juices and 1.0 – 6.0 x 10^2 CFU/ml for industrially packaged juices. Bacteria isolated include; *Bacillus* sp, *Micrococcus* sp, *Staphylococcus* sp, *Enterococcus* sp and *Escherichia coli*. *Bacillus* sp, *Staphylococcus* sp and *Enterococcus* sp were the highest occurring bacteria in locally processed juices while *Micrococcus* sp was the highest occurring bacterium in industrially processed samples and *Escherichia coli* was detected only in locally processed samples. The fungal isolates include; *Aspergillus* sp, *Penicillium* sp, *Saccharomyces cerevisiae* and *Trichoderma* sp. *Saccharomyces cerevisiae* was the highest occurring fungus in industrially processed juices; *Aspergillus* sp and *Penicillium* sp were the highest occurring fungi in locally processed samples while *Trichoderma* sp occurred only in locally processed samples.

Divyashree S. *et al.* (2013) enumerated and identified the microorganisms in fruit juices (sweet lime, orange and pineapple) selected from three different street vended shops (source A, B and C) in Mysore. The juices were analyzed for the microbial quality for type of organisms and number of colonies by serial dilution technique, pour plate method; Gram's staining method and staining for fungi; and physico-chemical properties. They showed that the pineapple juice from two sources was highly contaminated with bacterial pathogens (25×10^4 CFU/ml and 20×10^4 CFU/ml).

Gulzar Ahmad Nayik *et al.* (2013) assessed the microbial quality of fruit juices sold for immediate consumption in the markets of Kashmir valley. They were collected twelve fruit juice samples (3 from each apple, orange, pineapple and mango juices) from different markets and tested for their microbiological quality. Microbial quality was determined by enumerating the total viable count. About 25% of the samples (orange juice) did not comply with the standards of microbial quality as per the guidelines for microbiological quality of ready to eat foods while as apple, orange and pineapple juices complied with the standards. They observed the microbial load in orange juice was comparatively higher than that in the apple, pineapple and mango juice which had the microbial load within acceptable limits.

Muhammad Zahoor *et al.* (2013) investigated to determine the involvement of bacteria in the contamination of fruit juice and diseases caused by them. Nutrient agar plates were prepared and inoculated with selected juice samples. The plates were incubated for 24 hours at 37°C. After 24 hours, various colonies of microbes were produced at the surface of solid agar media in the plates and were identified by Gram staining and microscope. The pH of the juices was also determined. Various kinds of bacteria were detected in all the selected samples. The Cocci were observed in large quantity; Bacilli in moderate while Spirilla in minute quantity. The Cocci were mostly Gram Negative while the Bacilli were of Gram Negative type. The spirilla was absent in all the selected juice samples except mango.

Odu Ngozi Nma *et al.* (2013) analyzed some packaged fruit juice for microbiological quality using standard microbiological techniques. The fruit juices were purchased from street hawkers in Port Harcourt Metropolis, Nigeria. Total heterotrophic bacteria count of some of the packaged fruit juice samples ranged from $3.5x10^2$ to $7.1x10^3$ CFU/ml (for orange juice), $4.2x10^2$ to $6.6x10^4$ CFU/ml (for apple juice), and $3.0x10^2$ to $9.0x10^4$ CFU/ml (for pineapple juice). Total fungi count of some of the packaged fruit juice samples ranged from $1.5x10^2$ to $2.5x10^2$ CFU/ml (for orange juice), $2.0x10^2$ to $4.2x10^2$ CFU/ml (for apple juice) and $0.0x10^2$ to $2.2x10^2$ CFU/ml (for pineapple juice). Bacteria isolates obtained from the packaged fruit juices include; *Micrococcus* sp. (26.7%), *Flavobacterium* sp. (13.3%), *Bacillus* sp. (57.1%), *Lactobacillus* sp. (13.3%). The results also showed that of the fungi isolates obtained from packaged fruit juice, *Penicillum* sp. (57.1%) was predominant over *Saccharomyces* sp (42.9%). No coliform bacteria were observed in all packaged fruit juice samples. None of the fruit juice samples showed any growth of *Salmonella*, *Shigella* and *Vibrio* species. With the number of isolated bacteria and fungi from the different packaged fruit juice sold in Port Harcourt, it can be concluded that different bacterial and fungal species occur within fruits and materials used for the production of the juice as well as poor sanitation, extraction, raw material contaminations (often from insect damage), lack of both proper heat sterilization and adequate quality control during processing of fruit juice.

Asmamaw Leul *et al.* (2012) assessed bacteriological quality and safety of freshly squeezed mango and pineapple juices in Bahir Dar town, Ethiopia. They observed that aerobic mesophilic count of mango juice (4.76 log CFU/ml) was relatively higher than pineapple juice (4.21 log CFU/ml) across each juice house. The mean Staphylococcus aureus counts were 3.84 log CFU/ml in mango and 3.74 log CFU/ml in pineapple juices. Total coliform counts were in the range of 9.2 to > 1100 MPN/ml in mango and from < 3 to > 1100 MPN/ml in pineapple juices.

Rashed *et al.* (2012) investigated to resolve the microbiological attributes of the fruit juices collected from different areas around Dhaka

city. To check the total bacterial load, coliforms and staphylococci 26 vendor fruit juices and 15 packed juices were examined. Samples were found to harbor viable bacteria within the range between 10^2 -10^7 cfu/ ml. Thirty samples exhibited the presence of staphylococci. Total coliforms were detected in 31 samples within the range of 102 -106 cfu/ ml which were further detected as *Escherichia coli* and *Klebsiella* spp. Drug resistance among the isolates was found against ampicillin, ciprofloxacin, amoxicillin, erythromycin, chloramphenicol, ceftriaxone, piperaciline, trimethoprime-sulfomethoxazole, nalidixic acid and vancomycin.

Rashmi H Poojara *et al.* (2012) examined the microbiological profile of street foods. The street foods were classified on the basis of degree of processing as unprocessed, semi processed and processed foods. From each category two food stuffs were selected and three samples were collected for the assay. Apart from the food samples five, water and ice samples from the outlets were collected. Microbiological parameters assayed were *S aureus*, *V cholerae, Salmonella*, Total coliforms and *E coli*. Majority of the water and ice samples were not potable. Microbiological assay revealed that high temperature processing of foods make them microbiologically safe for human consumption by killing pathogenic organisms. The results reveal high degree of contamination in unprocessed foods and semi processed foods. Processed foods that have undergone processing at high temperatures are less contaminated. Water and ice used by street food vendors was microbiologically unsafe.

W. Braide *et al.* (2012) investigated the microbiological status of industrially processed fruit juices sold in Onitsha main market was determined using standard methods. Fourteen (14) brands of the samples consisting of seven single fruits and seven mixed fruit juices were repeatedly subjected to bacteriological and mycological screening for six months. Isolates were characterized colonially, microscopically and

biochemically, and their identity confirmed with reference to standard manuals. The processed fruit juices investigated showed high microbial loads consisting of bacteria such as *Bacillus* sp, *Staphylococcus* sp, *Enterococcus* sp *Pseudomonas* sp, *Micrococcus* sp and *Corynebacterium* sp. The Yeasts and moulds isolated are *Saccharomyces cerevisiae, Saccharomyces* var *ellipsoideus, Penicilluim caseicolum, Penicilium notatum, Rhizopus stolonifer* and an unidentified *Saccharomyces* species. Some of the isolates are normal commensals and or contaminants from the fruits and the environment. The presence of *Staphylococcus aureus, Bacillus* and *Penicillium* species portends health risk to consumers as some species produce potent toxins associated with food borne illnesses and mycotoxicoses. The Total Viable Count reveals a high microbial population across all the samples. These values are quite higher than the microbiological limits for fruit juices and nectars. Poor sanitary conditions and failure to adhere to good manufacturing practices during processing could influence the high microbial load.

Babalola Olubukola O. *et al.* (2011) isolated bacteria from the fruit juices were *Micrococcus* spp, *Flavobacterium, Streptococcus* spp, *Staphylococcus* sp., and *Bacillus* spp. They found that the same type of bacteria Bacillus sp, *Streptococcus* spp, *Staphylococcus* spp and *Micrococcus* spp are persistent isolates throughout the period of this study. They indicated that the bacteria are fruit borne rather than contaminants from air water and utensils alone. The isolates could be used as indicators of microbial quality.

Mahuya Mukhopadhyay *et al.* (2011) analyzed the microbial quality of the street vended juices sold in different places in Kolkata city, India. Total viable count, Yeast and mold count, Coliform count, *vibrio* count and *salmonella* count was analyzed using standard methods. They were

observed total viable counts (TVC) were high ranging from 265-700×10^4 CFU/1000ml. Yeast count varied between 1.8-360×10^4 CFU/1000ml where as Mould varies between 1.1-620×10^4 CFU/1000ml. Coliforms include both the presence of fecals (.5-45×10^4 CFU/1000ml) and non fecals (.15-76×10^4 CFU/1000ml). Again presence of *Vibrio* (1.1-536×10^4 CFU/1000ml) and *Salmonella* (.12-200×10^4 CFU/1000ml) were also observed in most of the tested samples.

Javid Ali *et al.* (2010) evaluated the microbiological quality of Un-Branded street vended andBranded juices sold in Peshawar City, Pakistan. These juices were analyzed microbiologically using standard microbiological methods. The analyzed parameters were Total Plate Count (TPC), Total Coliform Bacteria (TCB), Total Fecal Coliform Bacteria (TFC), *Esccheriachia coli* O157:H7 and Yeast and Mould. The Un-Branded juices (apple, banana, mango, orange, lemon and sugarcane) were microbiologically analyzed and showed that TPC were in the range of 9 x 109-4 x 10^4 cfu/ml, TCB were in the maximum value 210 (MPN/ml) for sugarcane juice and lowest 9.0 (MPN/ml) were calculated for orange juice. TFCB were absent in orange and lemon Juice, while apple, banana, mango and sugarcane juices were contaminated with TFC (MPN/ml) values 15, 23, 9.0 and 93 respectively. *E. coli* were present in apple, banana, mango and sugarcane juice, while it was absent in orange and lemon juices.

Sunday P. Ukwo *et al.* (2011) conducted a study to assess the microbiological quality and safety of fresh juices and edible ice sold in Uyo Metropolis. Fresh squeezed fruit juices of lime, lemon, pineapple and orange, vegetable juices of carrot, garlic and samples of edible ice were collected. All samples were analysed for total viable count (TVC), total coliform count (TCC), faecal coliform (FC) total Staphylococcal count (TSC), total Vibro count (Tvib.C) and the presence of *Salmonella*. Results indicated total viable count of all fruit juices were in the range of 4.90 –

6.81 (log cfu/100ml) and vegetable juices in the range of 5.42-6.73(log cfu/100ml), with significant load of coliforms, faecal coliforms, vibro and Staphylococcal counts. Qualitative counts showed the presence of coagulese positive Staphylococcal spp in almost all the samples, while *Salmonella* and *Vibro* were detected in pineapple, orange and carrot juices. All the edible ice samples collected from vendors indicated high microbial load of coliforms and staphylococcal counts. Findings indicate a huge load of pathogenic micro-organisms a fresh vended fruit and vegetable juices as well as edible ice used by the vendors.

Tasmina *et al.* (2010) conducted their study to assess the microbial quality of fresh and commercially packed available juices collected from different locations of Dhaka city. Standard culture techniques were followed to assess total viable count (TVC), total Staphylococcal count (TSC), total *Bacillus* count (TBC) and total fungal count (TFC) on different culture media and found the TVC varied from the range from 10^2 to 10^5 cfu/ ml with the highest of 2.4 x 10^5 cfu/ ml. A large number of Staphylococci and *Bacillus* was also found from several samples. Among total coliforms, *Klebsiella* spp., *Enterobacter* spp. along with *E. coli* was detected.

Tasnim F. *et al.* (2010) evaluated the nutritional and microbiological quality of industrially processed packed fruit juices of mango (*Mangifera indica*) and orange (*Citrus sinensis*) from nine different manufacturing companies in Dhaka City. The highest quantity of total sugar (17.62%) and reducing sugar (9.99%) was recorded in mango juices while the lowest in orange juices (10.41% and 2.24% respectively) of different companies. In this study, protein contents were comparatively higher in mango juices than in orange juices. The pH of all samples varied from 3.50±0.10 to 4.70±0.05. Vitamin C content was comparatively higher in mango juices. The levels of metals tested namely, arsenic, lead, copper and zinc in the juices were within the limits of Bangladesh Standard and Testing Institute (BSTI) for fruit juices. The microbiological qualities of all the products were within the limits of the Gulf standards (the

recommended Microbiological Standards for any fruit juice sold in the Gulf Region).

Ankur Titarmare *et al.* (2009) analysed microbiological quality of fresh squeezed juices of pineapple, sweet lime and vegetable juices sold by street vendors in Nagpur city. The samples were randomly collected from local vendors in the city. A total of 38 samples were analysed for total viable count, total and fecal coliforms, staphylococci on mannitol salt agar and *salmonella*. The total viable count in all the fruit and vegetable samples were in the range of $2.0x10^4$– $4.6x10^6$. They observed that there was no significant difference between the total coliform and staphylococcal counts in juices collected from different locations.

M. Shakir Uddin Ahmed *et al.* (2009) investigated Total viable bacterial counts, fungal counts, total coliform, faecal coliform and the presence of pathogenic microorganisms such as *E. coli, Bacillus cereus, Staphylococcus aureus, Salmonella, Streptococcus* were analyzed by standard methods they found total viable count of samples ranged from 3.00×10^2 to 9.60×10^8 and fungal counts ranged from 1.00×10^2 to 8.05×10^4. Out of 114 freshly prepared fruit juices samples collected 113 samples (99%) showed the presence of coliform and E. coli. The other bacteria like *B. cereus*, *Staphylococcus aureus,* Salmonella, Streptococcus were found in 64.91%, 6.14%, 7.89% and (5.26%) of the tested samples. The number and type of microorganisms recovered from the freshly squeezed fruit juices made them unsafe for drinking.

Tambekar D.H *et al.* (2009) reported that food borne illness associated with the consumption of fruit juices at several places in India and elsewhere. Total of 52 samples were analyzed and found *E.coli* (40%), followed by *Ps. aeruginosa* (25%), *Salmonella* spp. (16%), *Proteus* spp. (9%), *S. aureus* (6%), *Klebsiella* spp. (3%) and *Enterobacter* spp. (1%). The highest bacterial contamination was observed in sweet lemon (35%), pineapple (29%), and pomegranate, apple, and orange (12% each).

Uma Reddy B. *et al.* (2009) isolated fecal coliforms bacteria from the street vended fresh fruit juices sold along the road sides of Bellary city,

India and assessed its safety for human consumption. They found that, juice sample-1 was found to be most contaminated with a count of 1, 40,000 coliforms/ 100 ml, sample -3 with a count of 1,10,000 coliforms/ 100 ml, sample-2 was 1,500 coliforms/100ml. The least count of only 400 coliforms/100ml was observed in sample-4. Whereas, the water samples-1, 2 and 3 were also found to be totally contaminated with faecal coliforms with a count of 1,100 microbes per 100ml. But sample-4 contains only 28 microbes/ 100 ml. they also isolated other bacteria like *Klebsiella pneumoniae, Citrobacter freundii*, *Enterobacter aerogens* and *Escherichia coli* from the samples.

Durgesh P. Mahale *et al.* (2008) investigated total viable counts of all 30 samples were approximately log 6.5 cfu/100ml with significant load of coliforms, faecal coliforms, Vibrio and Staphylococcal counts. Qualitative counts showed the presence of coagulase positive *S.aureus* in 5 samples of sugarcane and 2 samples of carrot juice. Almost 70% of the ice samples collected from street vendors showed high microbial load ranging from log 5-8.5. .

Joy *et al.* (2006) aimed at examining the quality and safety of freshly squeezed fruit juices, in a metropolitan city (Visakhapatnam) in south India, based on standard techniques (e.g. culturing on selective media), showed that in most localities the street vended fruit juices remained hygienically poor since bacterial loads (Total viable counts and Total coliforms) on the whole are abnormally high (HVC 0.88-33.6×10^4 cfu/ 100 ml; TC 0.8-22.2×10^4 CFUs/ 100 ml). They suggested that regular monitoring of the quality of fruit juices for human consumption must be introduced to avoid any future pathogen outbreaks.

Oliveira ACG *et al.* (2006) determined heterotrophic bacteria, total and thermo-tolerant coliform counts, *Salmonella*, and parasites in the fresh sugarcane juice. 25% of samples showed poor sanitary conditions, with thermotolerant coliform levels higher than allowed by Brazilian standards. *Salmonella* spp. and parasites were absent in all samples. Thermo-tolerant coliforms were detected on the hands of 37% of juice handlers, and heterotrophic bacterial counts reached 2.0 x 10^3 cfu/per

hand. *E. coli* were detected in one hand sample, and no *Salmonella* spp. was detected.

CHAPTER 3

The present research work was conducted during January to June, 2017 in the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur. The detailed outline of Materials and Methods are given below.

3.1.1 Study area and study population

Forty (40) fresh fruit juice samples were collected from different areas around Dinajpur city of Bangladesh and brought to the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur for bacteriological analysis.

3.1.2 Laboratory preparation

All items of glassware's including test tubes, pipettes, cylinder, flasks, conical flasks, glass plate, slides and vials soaked in a household dishwashing detergent solution ('Trix, Recket and Colman Bangladesh Ltd.) for overnight, contaminated glassware's were disinfected in 2% sodium hypochloride solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly and finally sterilized either by dry heat at 160^0 C for 2 hours or by autoclaving for 15 minutes at 121^0 C under 15 lbs pressure per square inch. Autoclaved items were dried in a hot air oven over at 50^0 C. Disposable plastic were (micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50^0 C for future use.

3.1.3 Instrument and appliances

Phase contrast microscope, immersion oil, test tubes, petridish, cotton, hand gloves, plastic syringe (5 ml), micropipette (1 ml, 500 µl, 10-20 µl), glass slides, eppendorf tubes, magnifying glass, marker pen, ice-box, spirit lamp, balance, laminar flow, cover slips, inoculating loop, rack, autoclave, refrigerator, conical flask etc.

3.1.4 Media for culture

The media and reagents that have been used for this study are mentioned below.

3.1.4.1 Solid media

- Nutrient Agar (NA) base (Hi-media, India).
- Plate count agar (PCA) media (Hi-media, India).
- Eosin methylene blue (EMB) agar (Hi-media, India).
- MacConkey agar medium (Hi-media, India).
- Salmonella-Shigella (SS) agar (Hi-media, India).
- Mannitol Salt Agar (MSA)(Hi-media, India)
- Mueller Hinton Agar (Hi-media, India).

3.1.4.2 Liquid media

- Nutrient broth (Hi-media, India).
- 1% peptone water (Hi-media, India).

3.1.4.3 Media for biochemical test

- Triple Sugar Iron (TSI) agar slant (Hi-media, India).
- Motility, Indole, Urease (MIU) medium (Hi-media, India).
- Methyl Red-Voges Proskauer (MR-VP) broth, (Hi-media, India)

3.1.5 Reagents

The chemicals and reagents used during the study were-

- Gram's staining reagent: Crystal violet, Gram's iodine, Acetone and Safranine.
- Phosphate Buffered Saline (PBS)
- Physiological Saline Solution (PSS)
- Methylene Blue stain
- Voges-Proskauer (VP) Solution
- Indol Solution
- Methyl Red Solution
- Alpha-naphthol solution.
- Kovac's reagent.
- Ethyl alcohol (70% and 95%).
- Potassium- di-hydrogen phosphate (0.2M, KH_2PO_4 $2H_2O$)
- Di-sodium hydrogen phosphate (0.2M, $Na_2HPO_4 12H_2O$)
- Sugar media (Dextrose, Maltose, Lactose, Sucrose, and Mannitol) and other chemicals and reagents.

3.1.6 Antimicrobial Sensitivity Discs

To determine the drug sensitivity pattern of different bacteria isolate with different types of antimicrobial. Commercially available antimicrobial discs were used. The method allowed for the rapid determination of the efficacy of the drug by measuring the diameter of the zone of inhibition that result from different diffusion of the agent into the medium surrounding the disc. The followings are the

antibiotics that were tested against, the selected organism with their disc concentration.

Table No: 1. Antimicrobial agent with their disc concentration

S/N	Name of antibiotics	Disc concentration (µg/disc)	S/N	Name of antibiotics	Disc concentration (µg/disc)
1.	Ampicillin (AMP)	30 µg/disc	7.	Vancomycin (VA)	30 µg/disc
2.	Amoxicillin (AMX)	10 µg/disc	8.	Ciprofloxacin (CIP)	5 µg/disc
3.	Cloxacillin (CLO)	5 µg/disc	9.	Erythromycin (E)	15 µg/disc
4.	Gentamycin (GN)	5 µg/disc	10.	Levofloxacin (LF)	5 µg/disc
5.	Tetracycline (TE)	30 µg/disc	11.	Chloramphenicol (C)	30 µg/disc
6.	Azithromycin (AZM)	15 µg/disc			

Legend: µg = Microgram

3.2 Methods

3.2.1 Experimental layout

The experimental work was divided into two steps: The first step was performed for the Total Viable count (TVC), Total Coliform count

(TCC), Total Staphylococcal count and isolation & identification of organisms from the collected sample using cultural, staining and biochemical characteristics. The second step was conducted for the determination of antibiotic sensitivity and resistant pattern of isolated organisms from various samples by using different antibiotic discs available in the market. The layout of the diagrammatic illustration of the present study is shown in figure 1.

EXPERIMENTAL LAYOUT

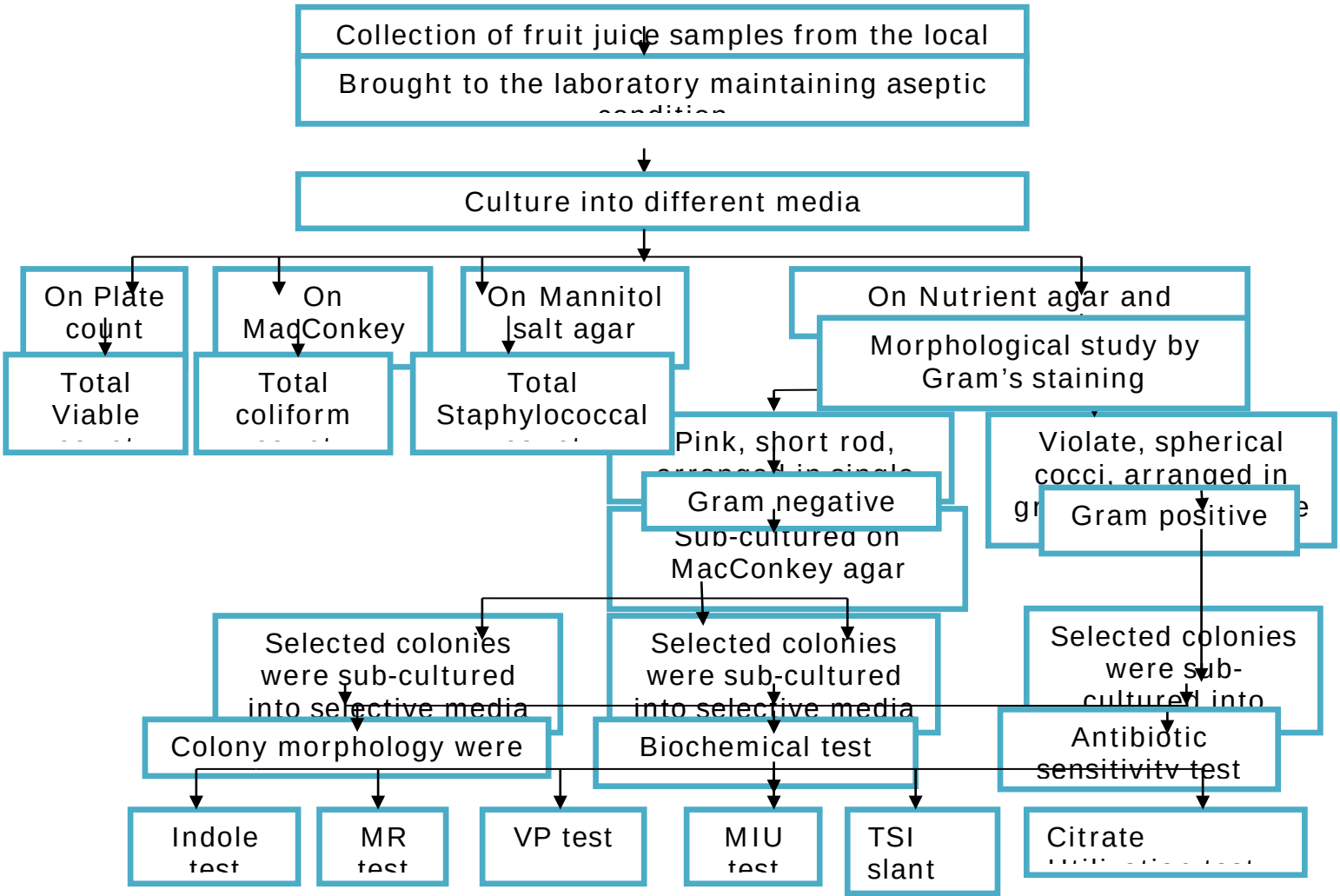


Fig: 1. The schematically illustration of layout of the experiment.

3.2.2 Preparation of culture media and biochemical media

All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer.

3.2.2.1 Liquid Media

3.2.3.1.1 Nutrient broth

Nutrient broth (NB) was used to grow the organisms from the samples collected from the study areas before performing biochemical test (Cheesebrough, 1985).

13.0 grams of Bacto-nutrient broth (Difco) was dissolved in 1000 ml of cold distilled water and heated up to boiling to dissolve it completely. The solution was then distributed in tubes, stoppered with cotton plugs and sterilized in the autoclave machine at l21°C and 15 pounds pressure per square inch for 15 minutes. The sterility of the medium was judged by incubating overnight at 37°C and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.2.2 Solid media

3.2.2.2.1 Plate Count Agar (PCA) medium

17.5 grams of plate count agar powder was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37^0C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.2.2.2 Nutrient Agar (NA) medium

28.0 grams of nutrient agar powder (Hi-media, India) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37^0C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.2.2.3 Mannitol Salt Agar (MSA) medium

111.02 grams of Mannitol salt agar power (Hi-media, India) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into

each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37^0 C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.2.2.4 Eosin Methylene Blue (EMB) agar medium

Eosin methylene blue (EMB) agar medium was used to observe the growth of *Escherichia coli* (Cheesebrough, 1985).
36.0 grams of EMB agar base (Hi-media, India) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in to sterile glass petridishes. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.2.2.5 MacConkey agar medium

51.50 grams of dehydrated Bacto-MacConkey agar (Difco) was suspended in 1000 ml of cold distilled water taken in a conical flask and was heated up to boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured sterile glass petridishes. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.2.2.6 Salmonella-Shigella (SS) agar medium

Selective medium for the isolation of *Salmonella* and *Shigella.* 63.0 grams SS agar powder was dissolved in 1000 ml of distilled water. It was mixed well until a homogeneous suspension is obtained. It was heated with frequent agitation and boiled for one minute. It did not sterilized by autoclaved. It was cooled to 45ºC and 50° C and distributed in Petri plates and allow the medium to solidify partially uncovered. (Hi-media and Leifson et al., 1935)

3.2.2.2.7 Mueller Hinton Agar

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. 38 grams of Mueller Hinton agar powder was suspended in 1000 ml of distilled water and mixed properly. It was heated agitating frequently and boiled for about one minute. It was dispensed and sterilized in autoclave at 116 - 121°C (15 lbs. sp) for 15 minutes. It was cooled to 45° or 50° C (Carter, 1979).

3.2.2.2.8 MIU medium

18.0 grams of MIU agar (Difco) was suspended in 950 ml of cold distilled water taken in a conical flask and heated up to boiling to dissolve the medium completely. 95 ml was despensed into flasks and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then was Cooled to about 50-55°C and aseptically 5ml was added of sterile 40% basal medium. After mixing were dispensed into sterile test tubes. Allow to cool in an upright position. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.3 Reagents preparation

3.2.3.1 Methyl Red-Voges Proskaure test

3.2.3.1.1 Methyl Red-Voges Proskaure broth

A quantity of 3.4 grams of Bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the

test tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and used for biochemical characterization or stored at 4°C in refrigerator for future use (Cheesbrough, 1984).

3.2.3.1.2 Methyl-Red (MR) solution

The indicator methyl red (MR) solution was prepared by dissolving 0.1 gm of Bacto methyl red (Difco) in 300 ml of 95% alcohol and diluting this to 500 ml with the addition of 200 ml of distilled water.

3.2.3.2 Voges-Proskauer (VP) solution

3.2.3.2.1 Alpha-naphthol solution

Alpha-naphthol solution was prepared by dissolving 5 grams of 1-naphthol in 100 ml of 95% ethyl alcohol.

3.2.3.2.2 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of potassium hydroxide crystals in 100 ml of cold distilled water.

3.2.3.3 Indole test

3.2.3.3.1 Kovac's reagent

This solution was prepared by mixing 25 ml of concentrated Hydrochloric acid in 75 ml of amyl alcohol and to this mixture 5 grams of paradimethyl-aminohenzyldehide crystals were added. This was then kept in a flask equipped with rubber cork for future use (Merchant and Packer, 1967).

3.2.3.3.2 Phosphate buffered saline (PBS) solution

For preparation of Phosphate buffered saline (PBS) solution, 8 gram of

sodium chloride (NaCl), 2.89 gram of disodium hydrogen phosphate (Na_2HPO_4, $12H_2O$), 0.2 gram of potassium chloride (KCl) and 0.2 gram of potassium hydrogen phosphate were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121 °C maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 (Cheesbrough, 1984).

3.2.4 Collection and transportation of fruit juice sample

A number of total forty (40) fresh fruit juice samples were collected directly from different areas around Dinajpur city. The samples were brought to the bacteriology laboratory, Department of Microbiology, HSTU, Dinajpur, in an ice box containing ice with necessary precautions and processed for the bacteriological examination.

3.2.5 Serial dilution of sample

Serial 10 fold dilutions of each of the fruit juice samples in a series of dilution tubes were prepared. At first for each of the juice samples 10 sterile test tubes were placed on a test tube holder rack containing 9 ml of 2% buffered peptone water.

1 ml juice was mixed with 9 ml of Phosphate buffer solution in the 1^{st} test tube in order to make 10^{-1} dilution. Then 1ml solution from 1^{st} test tube mixed with 2^{ndt} test tube, then from 2^{nd} test tube to 3^{rd} test tube and finally 9^{th} to 10^{th} test tube and 1ml discard from 10^{th} test tube by the help of pipette and in every steps mixing was done properly.

3.2.6 Enumeration of Total Viable Count (TVC)

For the determination of total viable bacterial count, 1 ml of each ten-fold dilution was transferred and spread on duplicate plate count agar using a fresh pipette for each dilution. The diluted samples were spread as

quickly as possible on the surface of the plate with a sterile glass spreader. One sterile spreader was used for each plate. The plates were then kept in an incubator at 37℃ for 24-48 hours. Following incubation, plates exhibiting 30-300 colonies were counted. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the total viable count. The total viable count was calculated according to ICMSF (1998). The results of the total bacterial count were expressed as the number of organism or colony forming units per ml (CFU/ml) of fruit juice sample.

3.2.7. Enumeration of Total Coliform Count (TCC)

For the determination of total coliform count o.1 ml of each ten-fold dilution was transferred to MacConkey agar. The diluted samples were spread as quickly as possible on the surface of the plate with a sterile glass spreader. One sterile spreader was used for each plate. The plates were then kept in an incubator at 37º C for 24 to 48 hours. Following incubation, plates exhibiting 30-300 colonies were counted. The average numbers of colonies in a particular dilution was multiplied by the dilution factor to obtain the total coliform count. The total coliform count was calculated according to ICMSF (1998). The results of the total coliform count were expressed as the number of organisms or colony forming units per ml (CFU/ml) of fruit juice samples.

3.2.8. Enumeration of Total Staphylococcal Count (TSC)

For the determination of total Staphylococcal count o.1 ml of each ten-fold dilution was transferred to Mannitol salt agar. The diluted samples

were spread as quickly as possible on the surface of the plate with a sterile glass spreader. One sterile spreader was used for each plate. The plates were then kept in an incubator at 37º C for 24 to 48 hours. Following incubation, plates exhibiting 30-300 colonies were counted. The average numbers of colonies in a particular dilution was multiplied by the dilution factor to obtain the total Staphylococcal count. The total Staphylococcal count was calculated according to ICMSF (1998). The results of the total staphylococcal count were expressed as the number of organisms or colony forming units per ml (CFU/ml) of fruit juice samples.

3.2.9 Isolation and identification of bacteria

The entire fruit juice samples were selected for bacteriological culture.

3.2.9.1 Culture of fruit juice sample

Media such as Nutient Agar (NA), MacConkey agar, Eosin Methylene Blue (EMB) agar, Mannitol Salt Agar (MSA) and Salmonella-Shigella (SS) agar were used.

3.3.9.2 Culture in ordinary media

Juice samples (n=20) were inoculated separately into ordinary media like nutrient agar media and were incubated at 37^{O}C for overnight. The colonies on primary cultures were repeatedly sub-cultured by streak plate method (Cheesbrough, 1984) until the pure culture with homogenous colonies were obtained.

3.2.9.3 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed culture was inoculated into nutrient agar media by streak plate technique to obtain isolated colonies as per:

Step-1: An inoculum was picked up with a sterile loop and spread on an area of the medium in the petridish.

Step-2: The loop was sterilized by being heated as red hot in a flame.

Step-3: The inoculum was spread over the reminder of the plate by drawing the cooled parallel line.

This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

3.2.9.2 Morphological characterization of organisms by Gram's staining method

The Gram's staining was followed to study the morphological and staining characteristics of bacteria and to provide information about the presumptive bacterial identification as per recommendation of Cowan and Steel (1979).

- A loopful of sterile distilled water was placed in the center of a clean sterile slide.
- A Small colony was picked up with a bacteriological loop and was mixed with distilled water on the slide.
- The colony was made to thin smear on a slide.
- The smears were fixed by air drying.
- 0.5% crystal violet solution was then applied on the smear for one minute.
- Gram's iodine was then added to act as mordant for one minute.
- Acetone alcohol was then added to decolorize for 1-2 seconds.
- Then the slide was washed with water.
- Safranine was added as counter stain and allowed for one minute.
- The slide was then washed with water.
- Then the slide was blotted with blot paper and was allowed to air dry. The slide was examined under microscope with high power objective (100X) using immersion oil.

Both of the gram positive and negative bacterial culture and mixed culture were selected.

3.2.9.3 Culture on differential Media

3.2.9.3.1 Mac-Conkey agar

Samples were sub-culture on Mac-conkey agar media and inocubated at $37^{O}C$ for overnight. After that Lactose fermenter (rose pink color colony) and lactose non fermenter (pale color colony) were selected.

3.2.9.3.2 Culture on selective media

3.2.9.3.2.1 Eosin Methylene Blue (EMB) agar:

Samples of positive lactose fermenter were taken and sub-culture on EMB agar media and incubated at 37^{O}C for overnight. Some EMB agar plate showed slightly circular colonies with dark center metallic sheen. Also in some EMB agar, the growth was indicated by smooth, characteristics mucoid colonies which are a consequence of the organism's abundant polysaccharide capsule.

3.2.9.3.2.2 Salmonella-Shigella (SS) agar

Sample of non lactose fermenter were taken and sub-culture on SS agar media and incubated at 37^{O}C for overnight, which after inoculation, raised, black centered, smooth round colony was present.

3.2.9.3.2.3 Mannitol Salt Agar (MSA)

Gram positive cultures were inoculated into Mannitol salt agar plates.

3.2.9.4 Microscopic study for identification of *E.coli, Salmonella* spp. *Staphylococcus* spp. *and Klebsiela* spp. suspected colonies by Gram's staining

Gram's staining was performed by taking colony from selected media to determine the size, shape, and arrangement of bacteria according to the methods described by Merchant and Packer (1967). Stained slides were examined under light microscope at 100 x magnification.

3.2.9.5 Identification of isolated organisms by different biochemical Tests:

Isolated organisms with supported growth characteristics of *E.coli. Salmonella* spp. *Staphylococcus* spp*. and Klebsiela* spp*.* were maintained in pure culture and subjected to biochemical test.

3.2.9.5.1 Procedure of Indole test

2 ml of peptone water was inoculated separately with 5 ml of culture of each of the isolated bacteria and incubated for 48 hours. 0.5 ml Kovac's reagent was added, shaked well and examined after 1 minute. A red colour ring at the top of the reagent indicated production of the indole by the organisms (Cowan, 1985).

3.2.9.5.2 Procedure of Methyl-Red (MR) test

The test was performed by inoculating separately a colony of the each of the isolated test organisms in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37^0C, a drop of methyl red solution was added. A positive methyl red test was shown by the appearance of a bright red colour. A yellow or orange colour was a negative test (Cowan, 1985).

3.2.9.5.3 Procedure of Voges-Proskauer (VP) test

2 ml of sterile glucose phosphate peptone water were inoculated separately with 5ml of each of the isolated organisms and incubated at 37^0 C for 48 hours. A very small amount (knife point) of creatine was added and mixed. 3 ml of 40% potassium hydroxide were added and shaked well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of a pink colour for positive cases. In negative cases there was no development of pink colour (Cowan, 1985).

3.2.9.5.4 Procedure of Motility, Indole, Urease (MIU) Test

MIU media were prepared in test tubes. Then the isolated organisms were inoculated separately into the media by stabbing method with the help of sterile straight wire. Then the test tubes were incubated 37^0 C overnight. Single stick that is no turbidity throughout the medium indicate gram negative organism (non motile) and turbidity throughout the medium indicate positive case (Cowan, 1985).

3.2.9.5.5 Procedure of Triple Sugar Iron (TSI) Test

Triple sugar iron contains three sugars (Glucose, Sucrose, and Lactose). At first TSI agar slant were prepared in a test tube. Then the isolated organisms were inoculated into the butt with a sterilized wire and on the slant with a wire loop producing zigzag streaking. The tubes were incubated for 24 hours at 37^0C.Yellow color of butt and slant of the test tube indicate fermentation of Glucose, Sucrose and Lactose fermentation and butt shows blacking indicate H_2S production (Cowan, 1985).

3.2.9.5.6 Citrate utilization test

Simmons citrate agar slants of 2 ml in each vials were prepared by autoclaving at 15 psi 121^0 C. Using sterile technique, small amount of each of the isolated bacteria from 24 hours old pure culture were inoculated separately into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37^0 C (Cappuccino & Sherman, 2005).

3.2.10 Maintenance of stock culture

During the experiment it was necessary to preserve the isolated organisms for longer periods. For this purpose the organisms from pure culture were inoculated into the tubes of nutrient agar slants and incubated at 37^0C for 24 hours. After the growth of organisms the tubes were sealed with paraffin wax and kept in the refrigerator at 4^0C following the procedures of (Choudhury *et al.,* 1985).

3.2.11 (20%) Sterile buffered glycerin

An amount of 20% of sterile buffered glycerin was made by mixing 20 parts pure glycerin and 80 parts PBS. Then a loopful of thick bacterial culture was mixed with 20% sterile buffered glycerin in small vials and was preserved at 20^0C. This method is more appropriate for preserving bacteria with no deviation of their original characters for several years (Buxton and Fraser, 1977).

3.2.12 Antibiotic sensitivity test and resistance pattern analysis

3.2.12.1 In vitro antibiotic sensitivity test

The method allowed for the rapid determination of the efficacy of the drugs by measuring the diameter of the zone of inhibition that resulted from different diffusion of the agent into the medium surrounding the disc.

In vitro antibiotic sensitivity tests were done using disc diffusion test following the method Kirby- Bauer (Bauer *et al.,* 1966). 1-2 ml of freshly growing broth culture were poured on Mueller Hinton agar plate and spread uniformly. Antibiotic discs were placed apart on to the surface of the inoculated plates aseptically with the help of a sterile forceps and incubated at 37 ^{0}C for 24 hours.

After incubation the plates were examined and the diameter of the zone of inhibition was measured. The diameter of the zone for individual antibiotic was recorded as sensitive, intermediate and resistant (According to EUCAST, 2015).

CHAPTER 4

RESULTS

4.1 Bacterial Counts

4.1.1 Total Viable Count (TVC)

A number of forty (40) fresh fruit juice samples were collected from different areas around Dinajpur city. All the samples were transported aseptically in the laboratory until use. Thereafter, the microbiological attribute were analyzed and studied comparatively.

The result presented in Table 2 showed the total viable bacterial load of forty (40) samples. The bacterial loads were not uniform and varied quite considerably. The total viable count varied with different types of juices that ranged from 1.8×10^4 to 5.6×10^7cfu/ml. The highest total viable count was found in Sugarcane juice (Sample, S-15) as 5.6×10^7cfu/ml collected from Gopalgonj bazar, Dinajpur and lowest total viable count was found in apple juice (Sample, S-20) as 1.8×10^4 cfu/ml collected from road side at HSTU campus.

4.1.2 Total Coliform Count (TCC)

Total coliform count of different fruit juice sample as ranged from 1.36×10^3 to 5.76×10^6 cfu/ml (Table 2). The highest total coiliform count was found in wood apple juice (Sample, S-29) as 5.76×10^6 cfu/ml collected from Nimnagor at Dinajpur town and lowest total coliform count was found in orange juice (Sample, S-9) as 1.36×10^3 cfu/ml collected from Modern more at Dinajpur town.

4.1.3 Total Staphylococcal Count (TSC)

Total Staphylococcal count of different fruit juice sample as ranged from 0 to 5.85×10^6 cfu/ml (Table 2). The height total Staphylococcal count was

found in Sugarcane juice (Sample, S-16) as 5.85×10^6cfu/ml collected from Bahadur bazar at Dinajpur town. *Staphylococcus* spp. was absent in 10 samples among collected 40 fresh fruit juice samples.

Table No: 2. Bacterial load in fresh fruit juice samples (n=40).

Sample No.	Types of juice	Sampling area	Total Viable Count(TVC) cfu/ml	Total Coliform Count(TCC) cfu/ml	Total Staphylococcal count(TSC) cfu/ml
S-1	Mango	Road side (HSTU Campus)	3.9×10^6	3.5×10^6	0
S-2	Mango	Modern more	3.4×10^7	3.9×10^4	4.2×10^3
S-3	Mango	Nimnagor	4.4×10^5	4.7×10^3	3.52×10^4
S-4	Mango	Suihari	3.4×10^6	2.5×10^6	3.3×10^5
S-5	Mango	Boromat	5.2×10^5	4.2×10^3	0
S-6	Mango	Bahadur Bazaar	2.8×10^7	3.6×10^5	3.94×10^3
S-7	Mango	Fulbari bus stand	5.4×10^4	2.3×10^3	3.16×10^4
S-8	Orange	Road side (HSTU Campus)	2.4×10^7	2.46×10^4	1.64×10^3
S-9	Orange	Modern more	5.4×10^5	1.36×10^3	1.86×10^2
S-10	Orange	Nimnagor	1.8×10^6	5.9×10^5	0
S-11	Orange	Suihari	2.7×10^7	2.7×10^4	1.74×10^5
S-12	Orange	Housing more	2.2×10^6	5.5×10^5	0
S-13	Sugarcane	Road side (HSTU Campus)	5.8×10^5	2.66×10^5	1.5×10^2
S-14	Sugarcane	Suihari	2.2×10^7	1.38×10^5	1.78×10^5
S-15	Sugarcane	Gopalgonj Bazaar	5.6×10^7	5.94×10^3	5.74×10^5
S-16	Sugarcane	Bahadur Bazaar	5.0×10^7	5.74×10^5	5.85×10^6
S-17	Sugarcane	Boromat	5.9×10^5	1.66×10^6	0
S-18	Sugarcane	Housing more	4.6×10^6	5.3×10^3	5.2×10^3

S-19	Sugarcane	Fulbari bus stand	4.2×10^5	4.3×10^4	2.1×10^5
S-20	Apple	Road side (HSTU Campus)	1.8×10^4	1.86×10^3	4.1×10^4

Sample No.	Types of juice	Sampling area	Total Viable Count(TVC) cfu/ml	Total Coliform Count(TCC) cfu/ml	Total Staphylococcal count(TSC) cfu/ml
S-21	Apple	Modern more	1.4×10^7	2.14×10^3	0
S-22	Apple	Nimnagor	2.5×10^6	2.9×10^4	4.84×10^5
S-23	Papaya	Road side (HSTU Campus)	1.6×10^5	1.92×10^3	2.1×10^4
S-24	Papaya	Gopalgonj bazaar	1.8×10^6	1.46×10^4	1.46×10^3
S-25	Papaya	Suihari	3.4×10^7	3.36×10^3	5.84×10^4
S-26	Papaya	Borobondor	2.2×10^5	2.9×10^5	0
S-27	Papaya	Fulbari bus stand	2.3×10^6	2.5×10^6	1.84×10^3
S-28	Wood apple	Road side (HSTU Campus)	2.24×10^6	5.8×10^4	5.8×10^4
S-29	Wood apple	Nimnagor	3.0×10^6	5.76×10^6	5.54×10^5
S-30	Wood apple	Suihari	1.8×10^7	5.3×10^5	3.96×10^3
S-31	Wood apple	Boromat	2.8×10^5	4.5×10^4	3.7×10^4
S-32	Wood apple	Bahadur Bazaar	3.4×10^5	4.8×10^6	0
S-33	Watermelon	Road side (HSTU Campus)	5.8×10^5	5.86×10^3	0
S-34	Watermelon	Modern more	5.2×10^7	5.54×10^5	5.96×10^3
S-35	Watermelon	Gopalgonj Bazaar	4.4×10^6	4.9×10^3	3.3×10^4
S-36	Watermelon	Borobondor	4.7×10^7	3.8×10^6	3.82×10^5
S-37	Watermelon	Housing more	3.8×10^5	4.3×10^4	4.3×10^3

S-38	Grape	Road side (HSTU Campus)	3.2×10^{6}	2.3×10^{2}	1.9×10^{2}
S-39	Grape	Modern more	4.7×10^{4}	1.9×10^{3}	0
S-40	Grape	Nimnagor	2.9×10^{7}	3.1×10^{4}	2.75×10^{3}

Table No: 3. Frequency of Occurrence of the bacteria Isolated from the Fruit Juice Samples (n=40).

Isolates	Number of Samples		
	Examined	Positive	Percentage (%
Escherichia coli	40	38	95%
Staphylococcus spp.	40	30	75%
Salmonella spp.	40	5	12.5%
Klebsiella spp.	40	4	10%

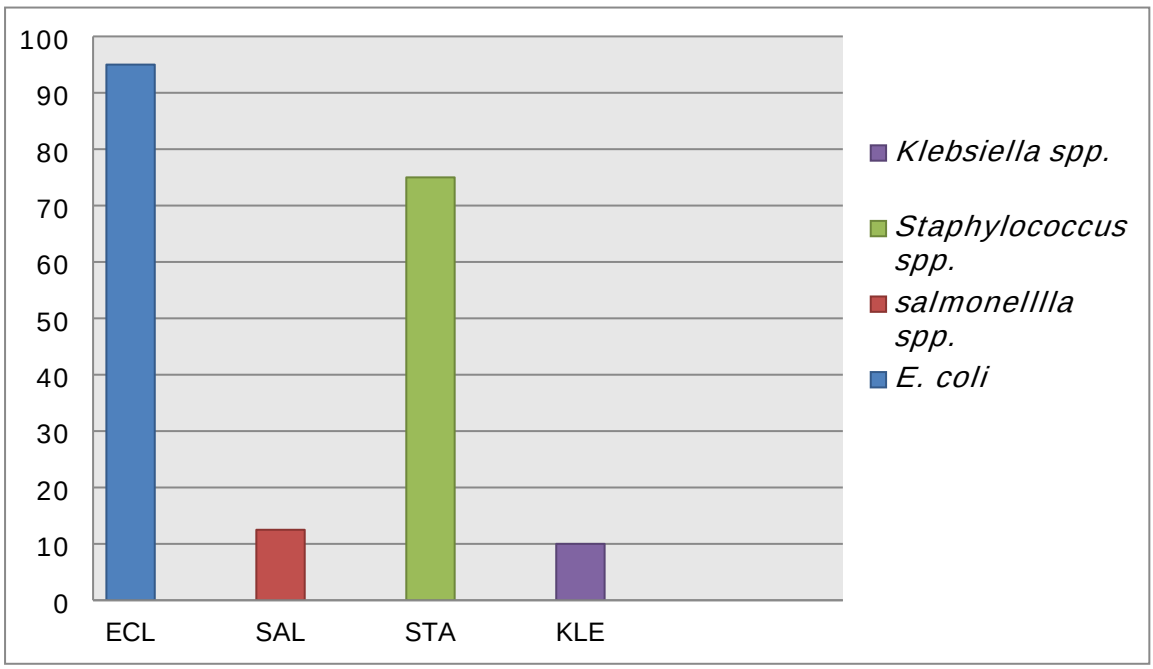


Fig: 2.Column diagram showing frequency of bacteria found in fresh fruit juice sample. The value of each bar is the frequency of each bacterium.

Legend:

ECL= *Escherichia coli.* KLE= *Klebsiella* spp.

SAL *= Salmonella* spp. STA= *Staphylococcus* spp.

Table No: 4. The recommended microbiological standards for any fruit juice; all numbers are as per ml of juice consumed (Gulf Standards, 2000).

Parameter	Total viable count (cfu/ml)	Coliform count(cfu/ml)	Fecal coliform count(cfu/ml)	Staphylococcal count(cfu/ml)
Maximum bacterial load anticipated	$5.0X10^3$	10	0	100
Maximum bacterial load permitted	$1.0X10^4$	100	0	$1.0x10^3$

4.2 Isolation & Identification of *Escherichia coli, Staphylococcus* spp., *Salmonella* spp. and *klebsiella* spp. by different bacteriological methods

E. coli, Salmonella spp., *Staphylococcus* spp. and *Klebsiella* spp. were frequently isolated from fresh fruit juice sample.

4.2. 1 Results of cultural examination

4.2.1.1 Ordinary media

4.2.1.1.1 Nutrient agar

Pale colorless colony was found (Plate-6).

4.2.1.2 For Gram negative cultures

4.2.1.2.1 Differential media

Gram negative pure cultures which were detected by gram's staining from nutrient agar were subcultures on MacConkey agar.

4.2.1.2.1 MacConkey agar

MacConkey agar plates streaked separately with the organisms from nutrient agar revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically.

The growth of lactose fermenting organisms was indicated by bright pink colored colonies of on MacConkey agar (Plate-7).

The growth of non lactose fermenting organisms was indicated by pale colored colonies of on MacConkey agar (Plate-7).

4.2.1.3 Selective media

4.2.1.3.1 Eosin Methylene Blue (EMB) agar

EMB agar plates streaked separately with the lactose fermenter organisms From MacConkey agar revealed the growth of *E.coli* and *Klebsiella* spp. bacteria after 24 hours of incubation at 37^0C aerobically.

The growth of *E.coli* was indicated by smooth, circular, black color colonies with metallic sheen on the agar plate (Plate-8).

The growth of *Klebsiella* spp. was indicated by smooth, Characteristics mucoid lactose-fermenting and pink colored colonies (Plate-9).

4.2.1.3.2 Salmonella-Shigella (SS) agar

SS agar plates streaked separately with the non lactose fermenting organisms from MacConkey agar revealed the growth of *Salmonella* spp. after 24 hours of incubation at 37^0C aerobically.

The growth of *Salmonella* spp. was indicated by smooth, Colorless; usually with black center (Plate-10).

4.2.3.2 For gram positive cultures

4.2.3.2.1 Mannitol Salt Agar (MSA)

Gram positive pure cultures from nutrient agar were subcultured directly on Mannitol salt agar. The organisms were observed as golden yellowish colonies on Mannitol salt agar (Plate-11).

4.2.2 Results of Gram's staining

The microscopic examination of Gram's stained smears from Nutrient agar revealed Gram- negative, pink colored and also Gram positive violate color organisms (Plate-12).

The microscopic examination of Gram's stained smears from EMB agar revealed Gram-negative, pink colored, small rod shaped *E.coli* arranged in single, pairs or short chain (Plate-13).

The microscopic examination of Gram's stained smears from SS agar revealed Gram- negative, pink colored, small rod shaped *Salmonella* spp. arranged in single, pairs or short chain (Plate-14).

The microscopic examination of Gram's stained smears from Mannitol salt agar revealed organisms were observed as Gram-positive cocci arranged in grape like clusters (Plate-15).

The microscopic examination of Gram's stained smears from EMB agar revealed Gram-negative, pink colored, small rod shaped *Klebsiella Spp*. arranged in single, pairs or short chain (Plate-16).

4.2.3 Results of biochemical tests

Isolated organisms were confirmed by different biochemical tests.

Table: 5. Identification of *E. coli* by biochemical tests

Biochemical test	Change of the media	Results	Plate no.
Indole test	Pink color ring	Positive	17
MR test	Red color	Positive	18
VP test	No color change	Negative	19
MIU test	Diffuse, hazy growth, slightly opaque media	Positive	20
Triple sugar iron (TSI) test	S-Yellow, B-Yellow	S-A, B-A, Gas (+), H_2S(-)	21
Citrate utilization test	No color change	Negative	22

Legends: (S= Slant, B= butt, A= Acid, (-) = Negative, (+) = Positive).

Table: 6. Identification of *Salmonella* spp. by biochemical test

Biochemical test	Change of the media	Results	Plate no.
Indole test	No color change	Negative	17
MR test	Red color	Positive	18
VP test	No color change	Negative	19
MIU test	Diffuse, hazy	Positive	20

	growth, slightly opaque media		
Triple sugar iron (TSI) test	S-Red, B-Yellow	S-Al, B-A, Gas (+), H_2S(+)	21
Citrate utilization test	No color change	Negative	22

Legends: (S= Slant, B= butt, A= Acid, Al= Alkaline, (-) = Negative, (+) = Positive).

Table: 7. Identification of *Staphylococcus* spp. by biochemical test

Biochemical test	Change of the media	Results	Plate no.
Indole test	No color change	Negative	17
MR test	Red color	Positive	18
VP test	Red color	Positive	19
MIU test	No color change	Negative	20
Triple sugar iron (TSI) test	S-Yellow, B-Yellow	S-A, B-A, Gas (-), H_2S(-)	21
Citrate utilization test	Prussian blue color	Positive	22

Legends: (S= Slant, B= butt, A= Acid, (-) = Negative).

Table: 8. Identification of *Klebsiella* spp. by biochemical test

Biochemical	Change of the	Results	Plate

test	media		no.
Indole test	No color change	Negative	17
MR test	No color change	Negative	18
VP test	Red color	Positive	19
MIU test	No color change	Negative	20
Triple sugar iron (TSI) test	S-Yellow, B-Yellow	S-A, B-A, Gas (+), H_2S(-)	21
Citrate utilization test	Prussian blue color	Positive	22

Legends: (S= Slant, B= butt, A= Acid, (-) = Negative, (+) = Positive).

4.3 Results of antibiotic sensitivity test and resistance pattern of isolated bacteria

The isolated bacterial pathogens were selected randomly for the antibiotic sensitivity test to detect resistance pattern against commonly used antibiotic. The results of sensitivity against antibiotic discs (zone of inhibition) were categorized as resistance, intermediate, sensitive. The results of antibiotic sensitivity are given in the table 9.

Table No: 9. Result of antibiotic sensitivity tests of the isolated bacteria obtained from fruit juice sample.

Antibiotics		*E coli* (n=38)		*Salmonella* (n=5)		*Staphylococcus* (n=30)		*Klebsiella* (n=4)	
		Zone size (mm)	Outcome	Zone size (mm)	Outcome	Zone size (mm)	Outcome	Zone size (mm)	Outcome
AMP	30 µg/disc	0	R (100%)	0	R (100%)	0	R (87%)	0	R (100%)
		-	I (0%)	-	I (0%)	-	I (0%)	-	I (0%)
		-	S (0%)	-	S (0%)	30	S (13%)	-	S (0%)
AMX	10 µg/disc	ND	ND	ND	ND	0	R (93%)	0	R (75%)
		-	-	-	-	-	I (0%)	-	I (0%)
		-	-	-	-	23	S (7%)	23	S (25%)
GN	5 µg/disc	0	R (5%)	ND	ND	ND	ND	ND	ND
		-	I (0%)	-	-	-	-	-	-
		20	S (95%)	-	-	-	-	-	-
CLO	5 µg/disc	0	R (89%)	ND	ND	ND	ND	ND	ND
		21	I (11%)	-	-	-	-	-	-
		-	S (0%)	-	-	-	-	-	-
TE	30 µg/disc	0	R (5%)	-	R (0%)	ND	ND	-	R (0%)
		13	I (5%)	18	I (40%)	-	-	13	I (25%)
		22	S (90%)	21	S (60%)	-	-	17	S (75%)
AZM	15	ND	ND	-	R (0%)	-	R (0%)	-	R (0%)

	µg/disc	-	-	-	I (0%)	16	I (13%)	-	I (0%)
		-	-	15	S (100%)	21	S (87%)	19	S (100%)

Antibiotics		*E coli* (n=38)		*Salmonella* (n=5)		*Staphylococcus* (n=30)		*Klebsiella* (n=4)	
		Zone size (mm)	Outcome	Zone size (mm)	Outcome	Zone size (mm)	Outcome	Zone size (mm)	Outcome
VN	30 µg/disc	ND	ND	9	R (20%)	ND	ND	ND	ND
		-	-	-	I (0%)	-	-	-	-
		-	-	17	S (80%)	-	-	-	-
CIP	5 µg/disc	0	R (58%)	-	R (0%)	ND	ND	ND	ND
		17	I (26%)	-	I (0%)	-	-	-	-
		23	S (16%)	33	S (100%)	-	-	-	-
E	15 µg/disc	ND	ND	ND	ND	-	R 0%)	ND	ND
		-	-	-	-	18	I (20%)	-	-
		-	-	-	-	24	S (80%)	-	-
LF	5 µg/disc	ND	ND	ND	ND	9	R (7%)	ND	ND
		-	-	-	-	-	I (0%)	-	-
		-	-	-	-	20	S (93%)	-	-
C	30 µg/dis	ND	ND	ND	ND	ND	ND	-	R (0%)
		-	-	-	-	-	-	-	I (0%)

	c	-	-	-	-	-	-	21	S (100%)

Legends: (AMP= Ampicillin, AMX= Amoxicillin, GN= Gentamycin, CLO= Cloxacillin, TE= Tetracycline, AZM= Azithromycin, VN= Vancomycin, CIP= Ciprofloxacin, E= Erythromycin, LF = Levofloxacin, C= Chloramphenicol, S= Sensitive, I= Intermediate, R= Resistant, ND= Not done, %= Percentage).

4.3.1 Antibiotic sensitivity test of *E. coli*

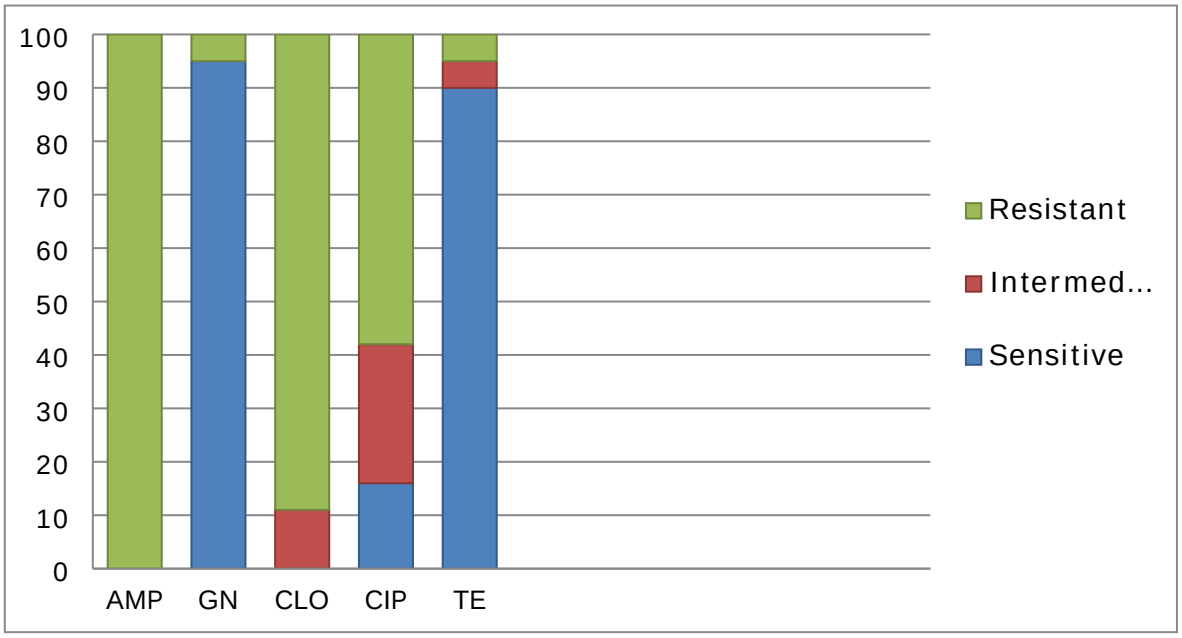
The antibiotic study revealed that the isolated *E.coli* were sensitive to Gentamycin (95%), Ciprofloxacin (16%) and Tetracycline (90%). The isolates were found to be resistant to Ampicillin (100%), Ciprofloxacin (58%) and Cloxacillin (89%). 11%, 26%, 5% isolates were intermediate sensitive to Cloxacillin, Ciprofloxacin and Tetracycline respectively.

Table No: 10. Results of antibiotic sensitivity test of *E. coli* (n =38)

Antibacterial agents	Disc concentration (µg /disc)	No. and Percentages (%) of isolates		
		Sensitive	Intermediate	Resistance
Ampicillin	30µg	(0) 0%	(0) 0%	(38) 100%
Gentamycin	5µg	(36) 95%	(0) 0%	(2) 5%
Cloxacillin	5µg	(0) 0%	(4) 11%	(34) 89%

Ciprofloxacin	5µg	(6) 16%	(10) 26%	(22) 58%
Tetracycline	30µg	(34) 90%	(2) 5%	(2) 5%

Fig.3. Column diagram presenting antibiotic sensitivity test of



isolated *E. coli*

Legends: (AMP=Ampicillin, GN=Gentamycin, CLO=Cloxacillin, CIP=Ciprofloxacin, TE=Tetracycline).

4.3.2 Antibiotic sensitivity test of *Salmonella* spp.

The antibiotic study revealed that the isolated *Salmonella* spp. were sensitive to Ciprofloxacin (100%), Azithromycin (100%), Vancomycin (80%) and Tetracycline (60%). The isolates were found to be resistant to Ampicillin (100%) and Vancomycin (20%). 40% isolates were intermediate sensitive to Tetracycline.

Table No: 11. Results of antibiotic sensitivity test of *Salmonella* spp. (n=5)

Antibacterial agents	Disc concentration (µg /disc)	No. and Percentages (%) of isolates		
		Sensitive	Intermediate	Resistance
Azithromycin	15µg	(5) 100%	(0) 0%	(0) 0%
Ampicillin	30µg	(0) 0%	(0) 0%	(5) 100%
Vancomycin	30µg	(4) 80%	(0) 0%	(1) 20%

Ciprofloxacin	5µg	(5) 100%	(0) 0%	(0) 0%
Tetracycline	30µg	(3) 60%	(2) 40%	(0) 0%

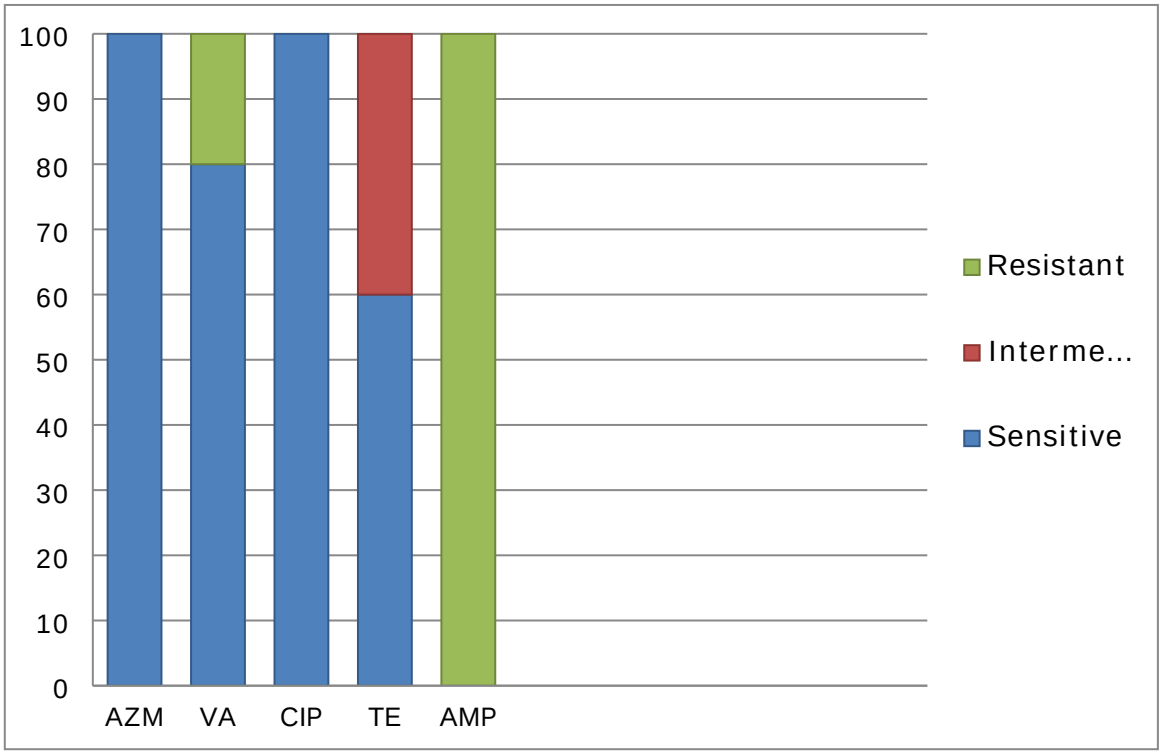


Fig. 4. Column diagram presenting antibiotic sensitivity test of isolated *Salmonella* spp.

Legends: (AZM=Azithromycin, VA=Vancomycin, CIP=Ciprofloxacin, TE=Tetracycline, AMP=Ampicillin).

4.3.3 Antibiotic sensitivity test of *Staphylococcus* spp.

The antibiotic study revealed that the isolated *Staphylococcus* spp. were sensitive to Azithromycin (87%), Ampicillin (13%), Levofloxacin (93%) and Erythromycin (80%). The isolates were found to be resistant to Ampicillin (87%) and Amoxicillin (93%). 13% and 20% isolates were intermediate sensitive to Azithromycin and Erythromycin respectively.

Table No: 12. Results of antibiotic sensitivity test of *Staphylococcus* spp. (n=30)

Antibacterial agents	Disc concentration (µg /disc)	No. and Percentages (%) of isolates		
		Sensitive	Intermediate	Resistance

Ampicillin	30µg	(4) 13%	(0) 0%	(26) 87%
Amoxicillin	10µg	(2) 7%	(0) 0%	(28) 93%
Azithromycin	15µg	(26) 87%	(4) 13%	(0) 0%
Levofloxacin	5µg	(28) 93%	(0) 0%	(2) 7%
Erythromycin	15µg	(24) 80%	(6) 20%	(0) %

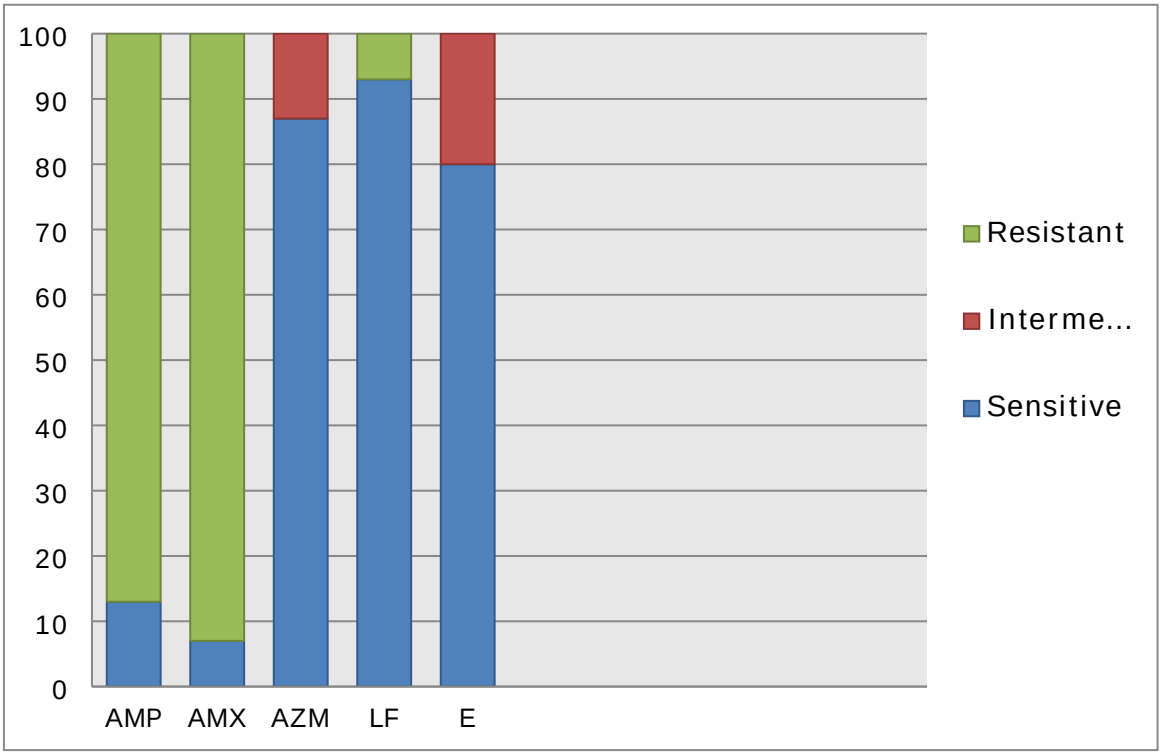


Fig: 5. Column diagram presenting antibiotic sensitivity test of *Staphylococcus* spp. Legends: (AMP=Ampicillin, AMX=Amoxicillin, AZM=Azithromycin, LF=Levofloxacin, E=Erythromycin).

4.3.4 Antibiotic sensitivity test of *Klebsiella* spp.

The antibiotic study revealed that the isolated *Klebsiella* spp. were sensitive to Chloramphenicol (100%), Tetracycline (75%), Azithroycin (100%) and found to be resistant to Ampicillin (100%), Amoxicillin 75%). 25% of the isolates were intermediate sensitive to Tetracycline.

Table No: 13. Results of antibiotic sensitivity test of isolated *Klebsiella* spp. (n=4)

Antibacterial	Disc	No. and Percentages (%) of isolates

agents	concentration (µg /disc)	Sensitive	Intermediate	Resistance
Ampicillin	30µg	(0) 0%	(0) 0%	(4) 100%
Amoxicillin	10µg	(1) 25%	(0) 0%	(3) 75%
Tetracycline	30µg	(3) 75%	(1) 25%	(0) 0%
Chloramphenicol	30µg	(4) 100%	(0) 0%	(0) 0%
Azithromycin	15µg	(4) 100%	(0) 0%	(0) 0%

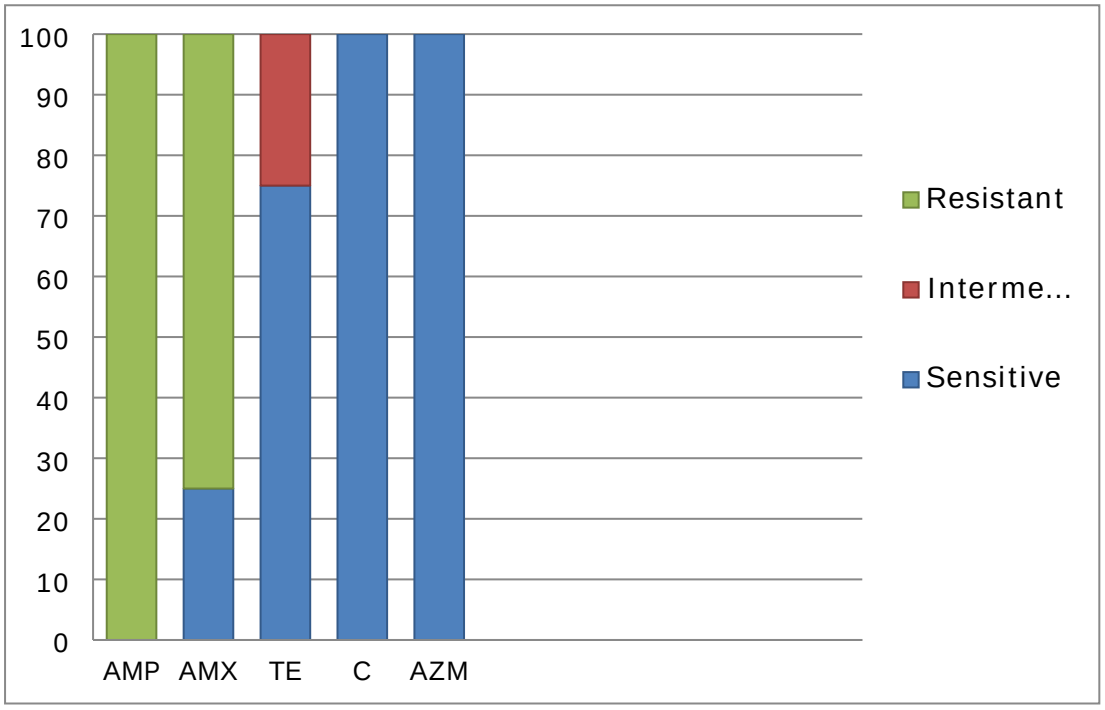


Fig.6. Column diagram presenting antibiotic sensitivity test of isolated *Klebsiella* spp.

Legends: (AMP=Ampicillin, AMX=Amoxicillin, TE=Tetracycline, C=Chloramphenicol, AZM=Azithromycin).

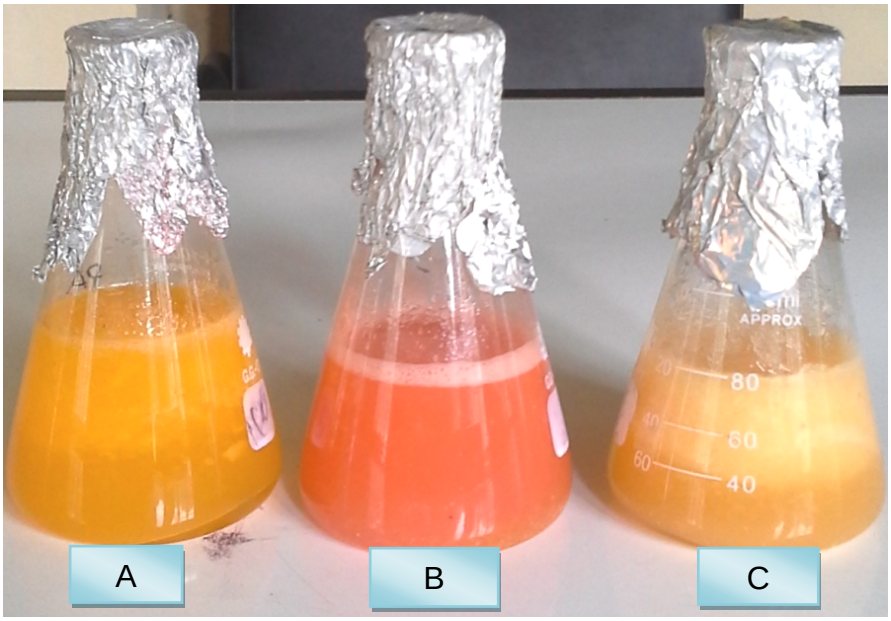


Plate 1: Fresh fruit juice samples. (A= Papaya, B=Watermelon C=Wood apple)

Plate 2: Ten fold dilution of fruit juice sample.

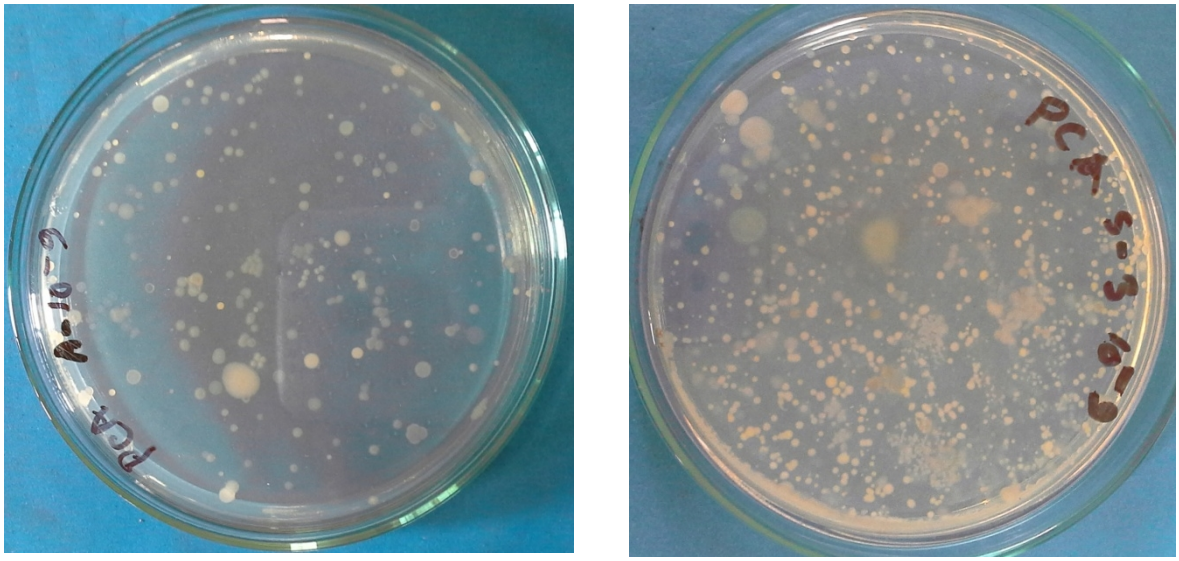


Plate 3: Colony of bacteria in Plate count agar for total viable count (TVC)

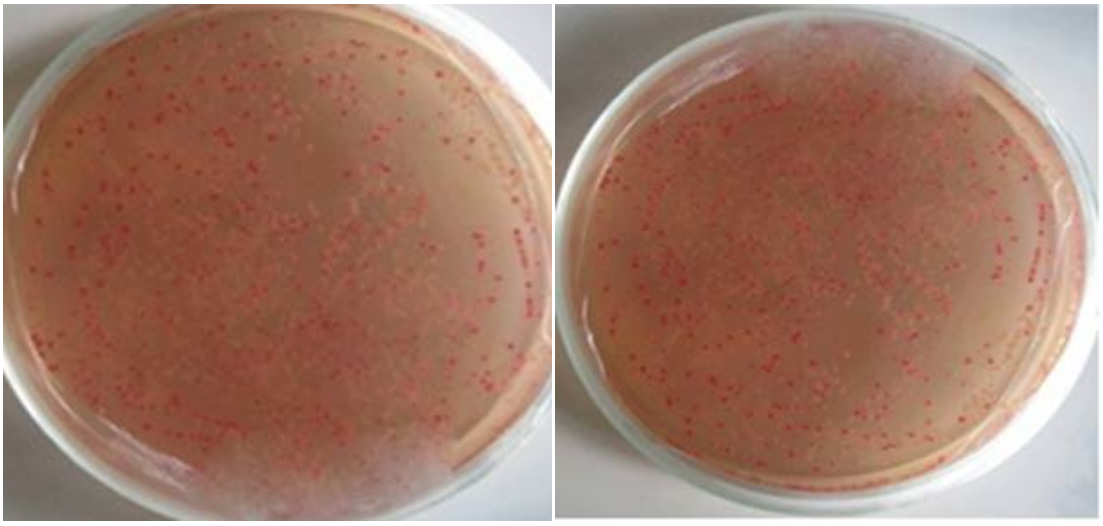


Plate 4: Colony of bacteria in MacConkey agar for total coliform count (TCC)

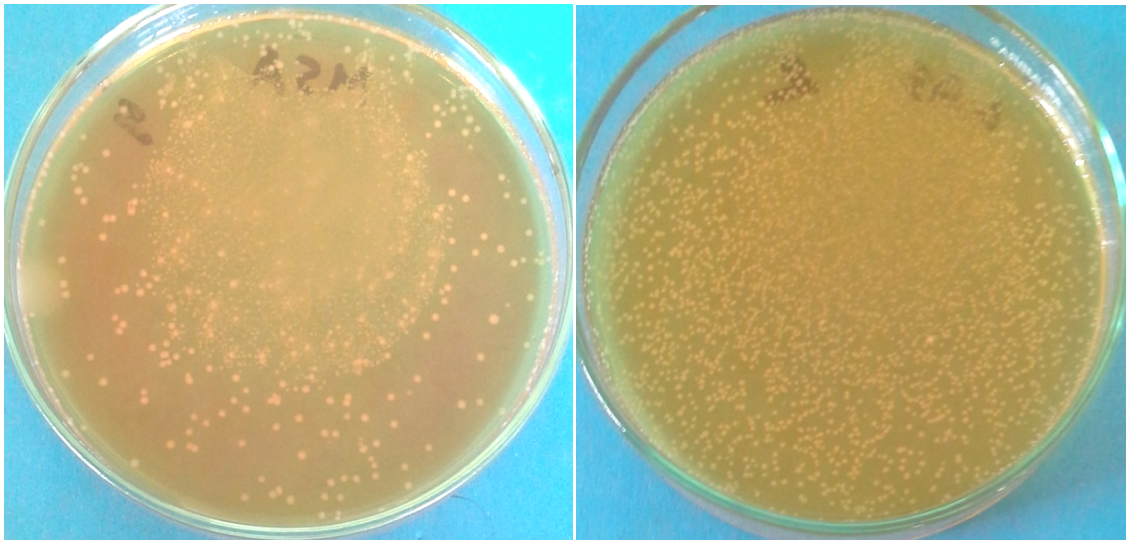


Plate 5: Colony of bacteria in Mannitol salt agar for total Staphylococcal count (TSC)

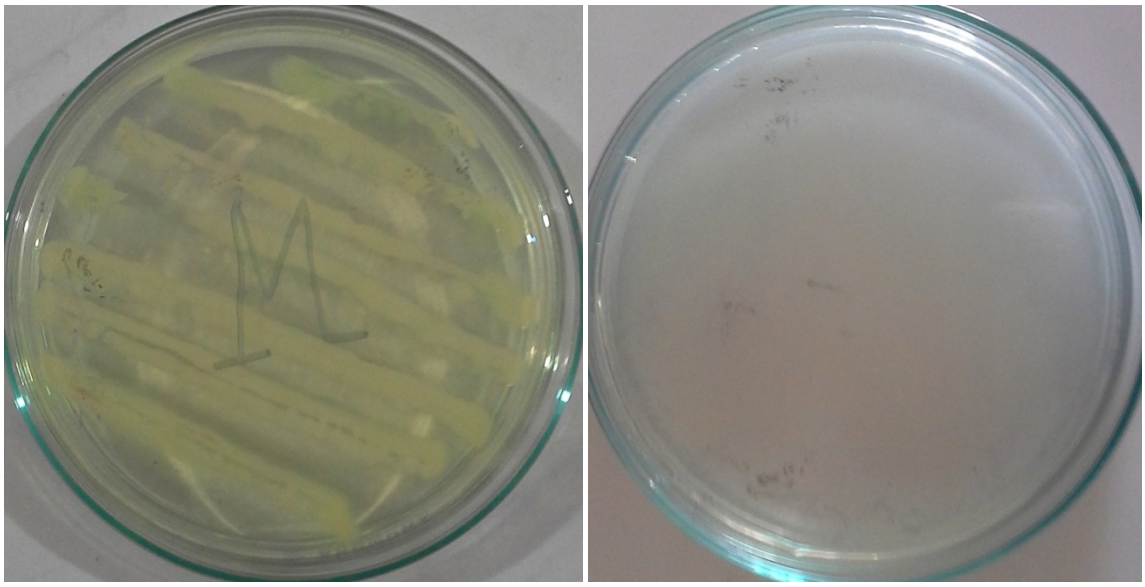


Plate 6: Bacteria produced pale colorless colonies on Nutrient agar (left) and uninoculated control (right)

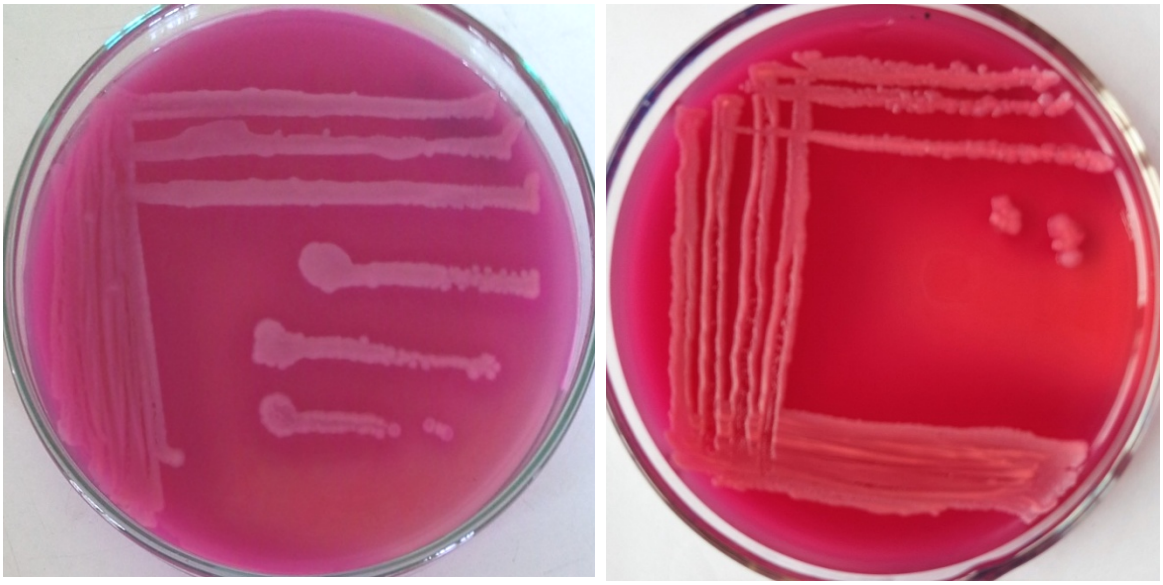


Plate 7: Lactose fermenting organisms produce bright pink colored colonies (Right) and non lactose fermenting organisms produce pale colored colonies (Left) of on MacConkey agar.

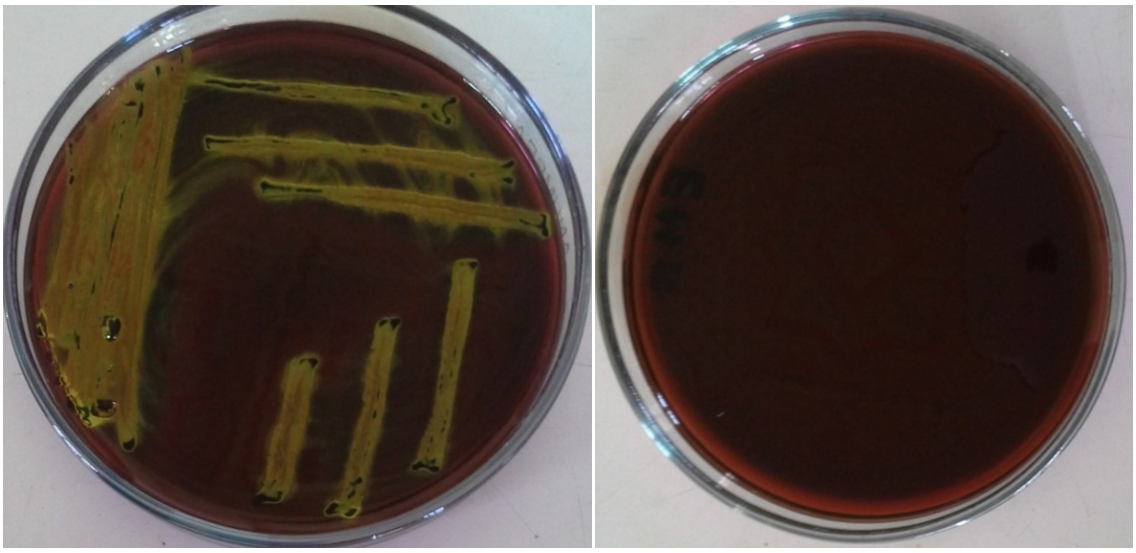


Plate 8: Metallic sheen produced by *E. coli* on EMB agar (left) and uninoculated control (right).

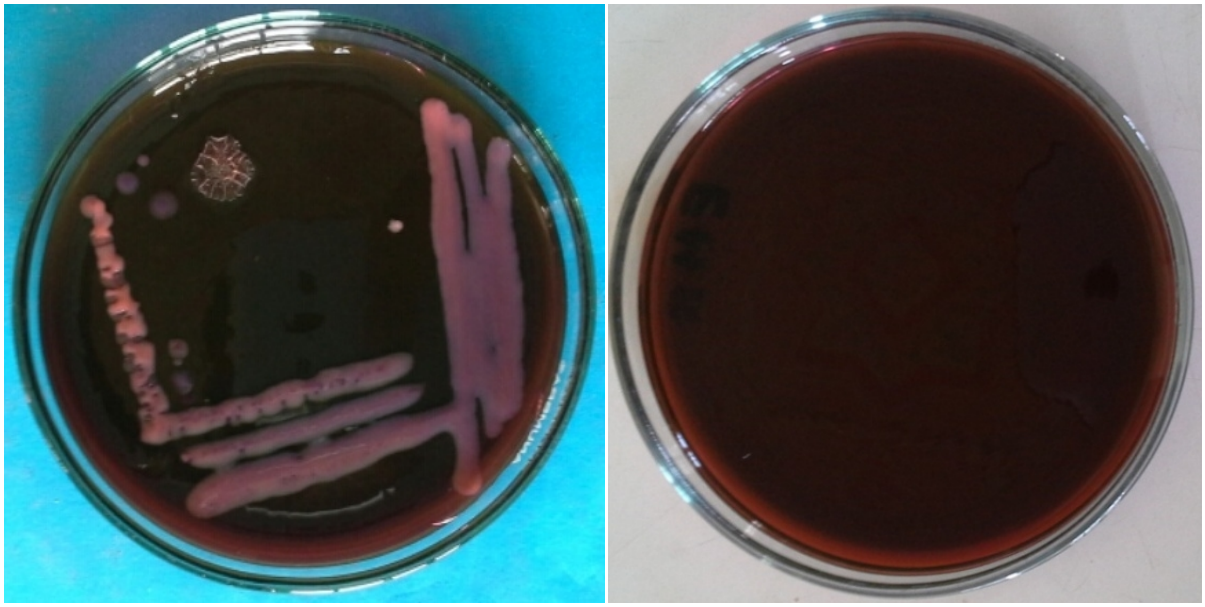


Plate 9: Pink colonies produced by *Klebsiella* on EMB agar (left) and uninoculated control (right).

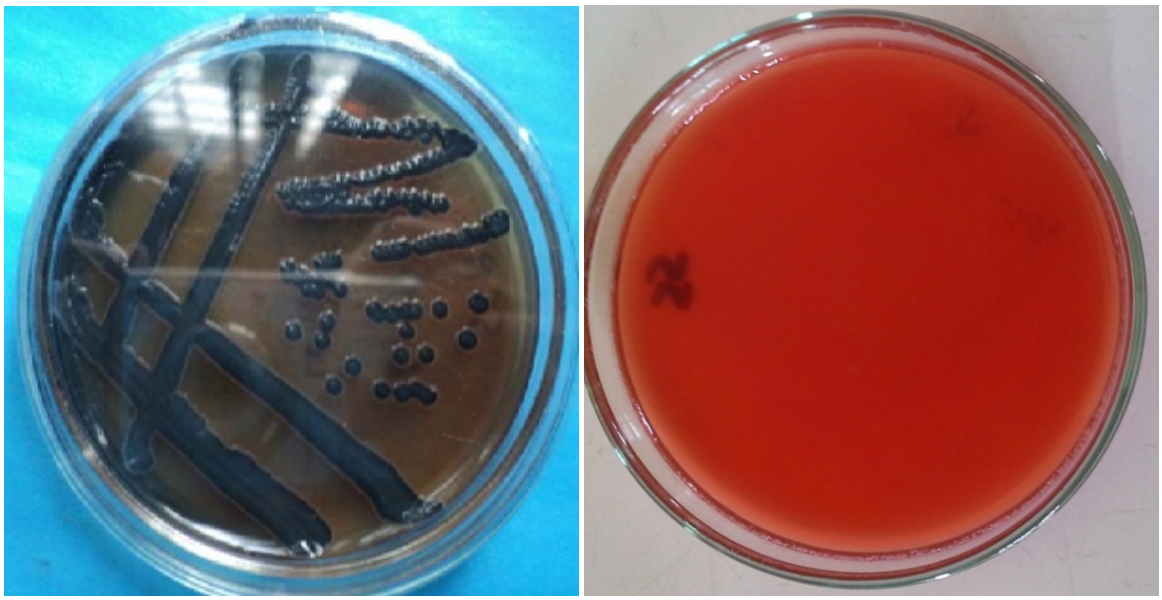


Plate 10: Black center colonies produced by *Salmonella* on SS agar (left) and un-inoculated control (right).

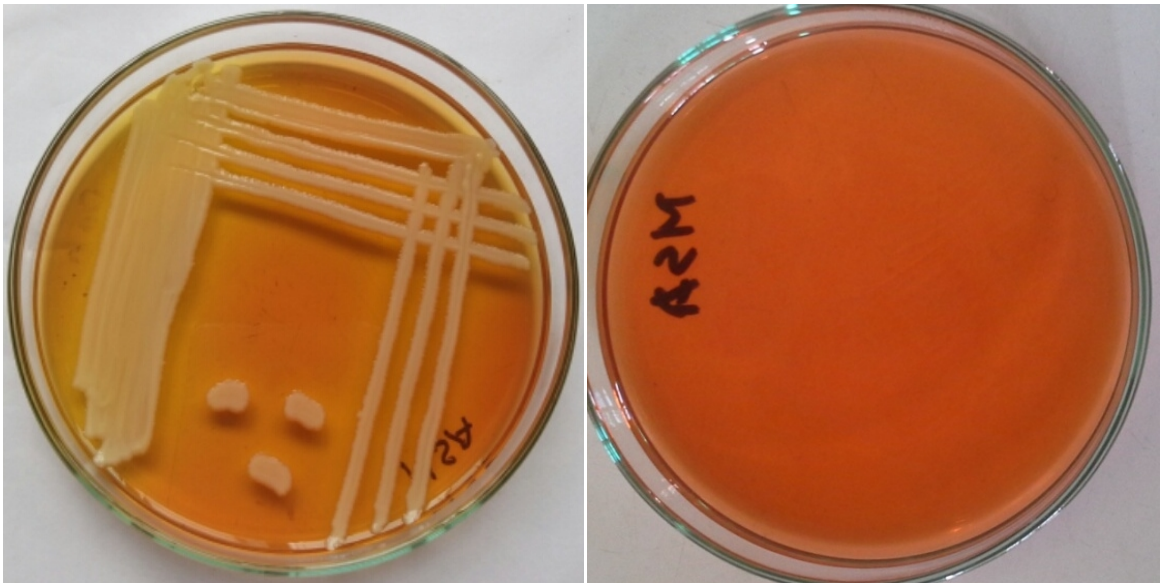


Plate 11: Golden yellowish colony produced by *Staphylococcus* on MSA agar (left) and uninoculated control (right).

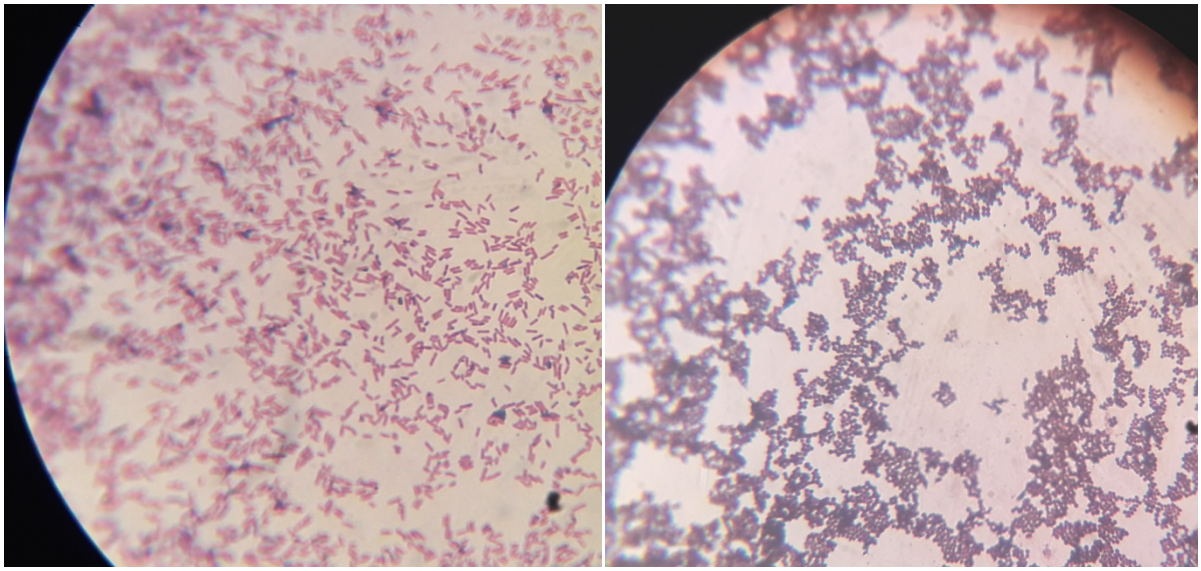


Plate 12: Gram's stained smear from nutrient agar revealed Gram negative bacteria, pink color (Left) and Gram positive bacteria, Violate color (Right)

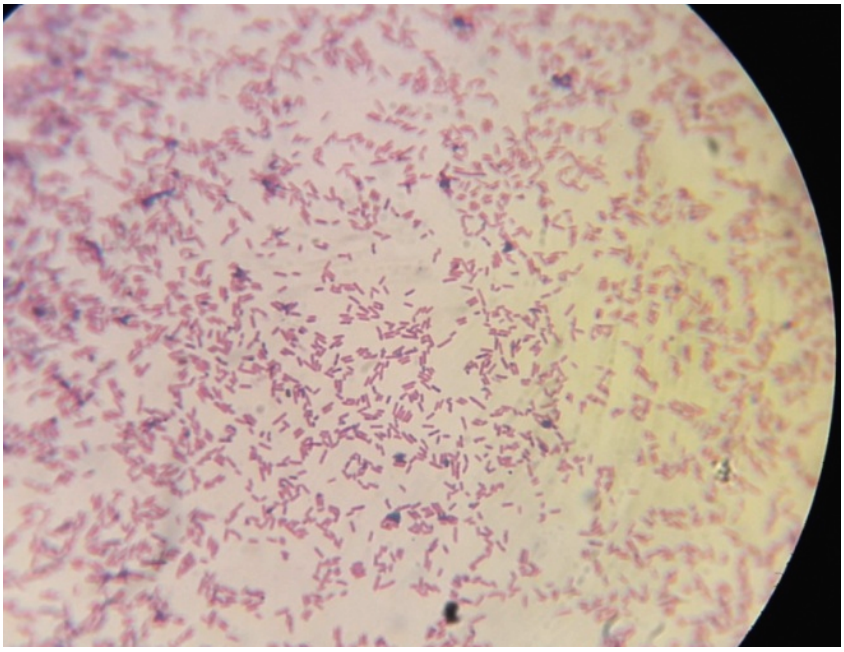


Plate 13: Gram's stained smears from EMB agar revealed Gram-negative, pink colored, small rod shaped *E.coli* arranged in single, pairs or short chain (100x magnification).

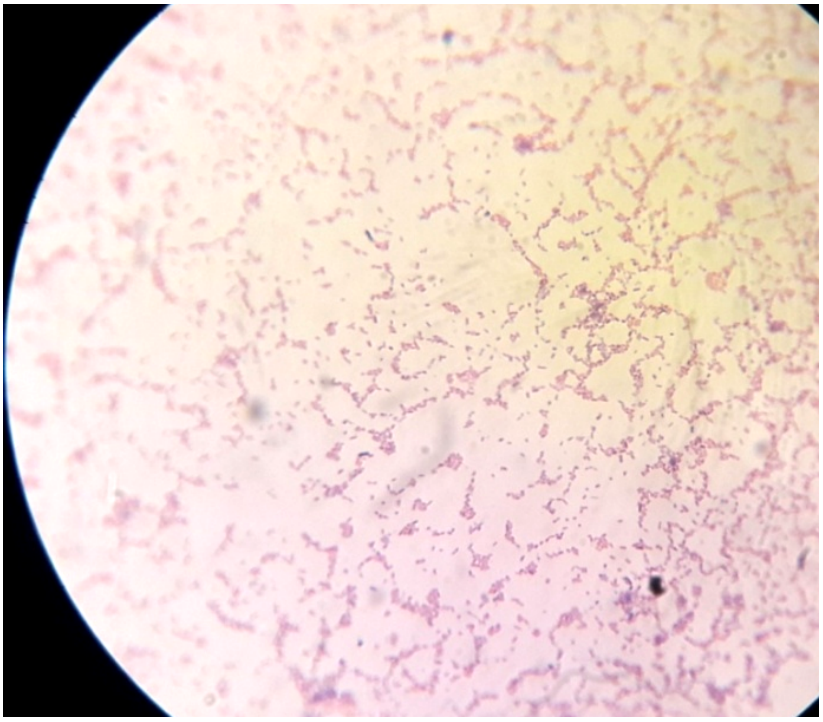


Plate 14: Gram's stained smears from SS agar revealed Gram-negative, pink colored, small rod shaped *Salmonella* spp. arranged in single, pairs or short chain (100x magnification)

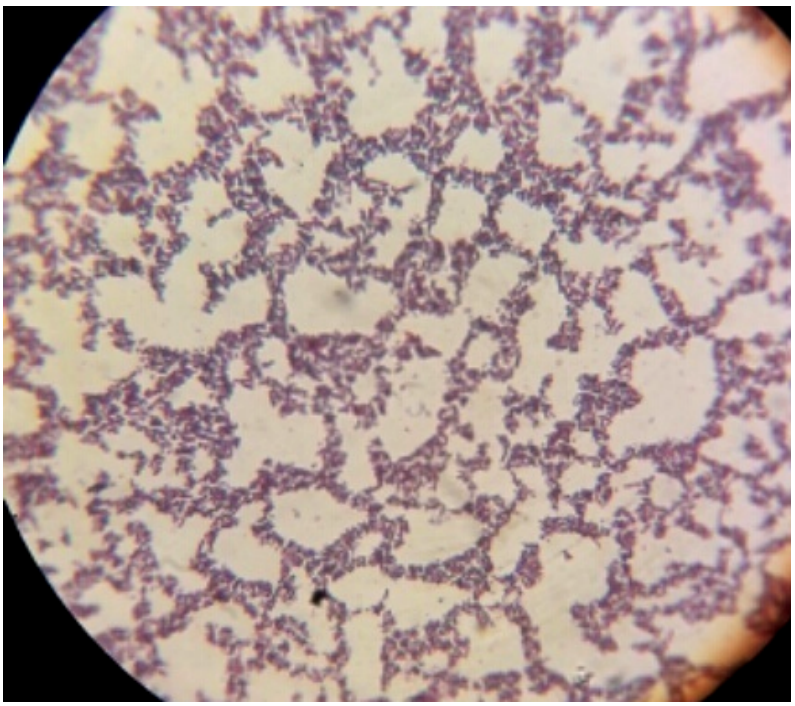


Plate 15: Gram's stained smears from Mannitol salt agar revealed Gram-positive cocci arranged in grape like clusters *Staphylococcus* spp. (100x magnification).

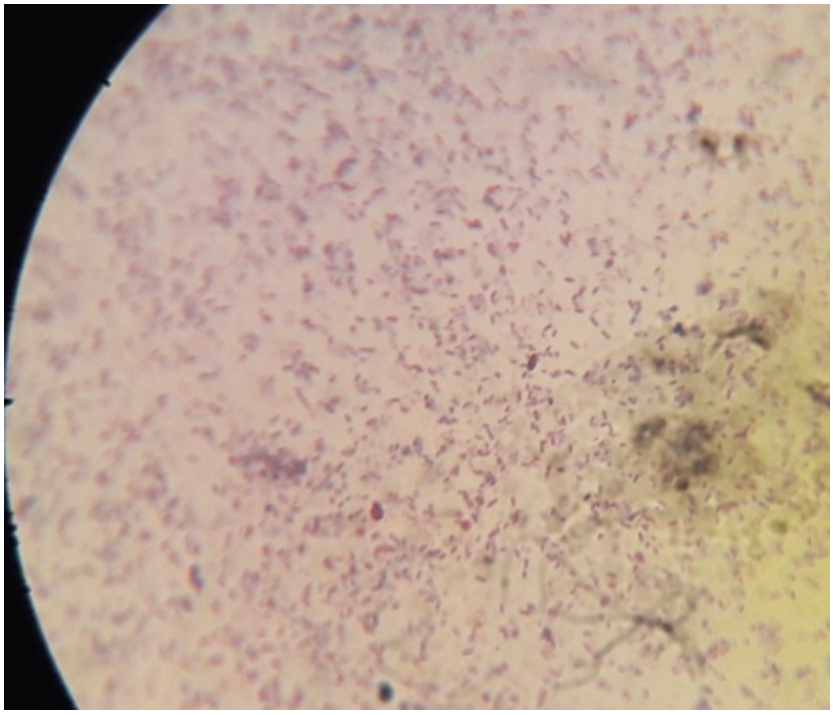


Plate 16: Gram's stained smears from EMB agar revealed Gram-negative, pink colored, small rod shaped *Klebsiella Spp*. arranged in single, pairs or short chain (100x magnification)

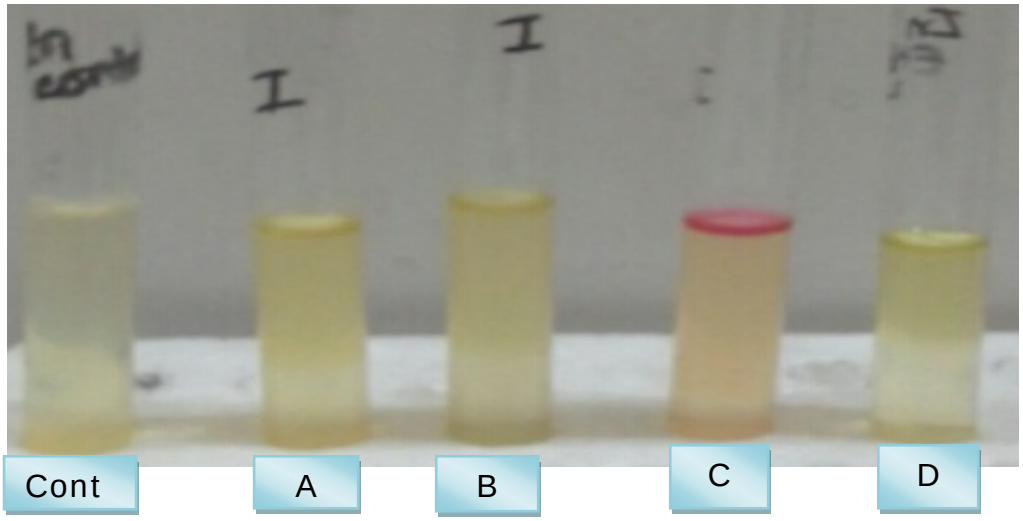


Plate 17: Indole test results (Right) A= *Salmonella* (Negative) B= *Klebsiella* (Negative), C= *E.* coli (Positive), D= *Staphylococcus* (Negative) and uninoculated control (Left).

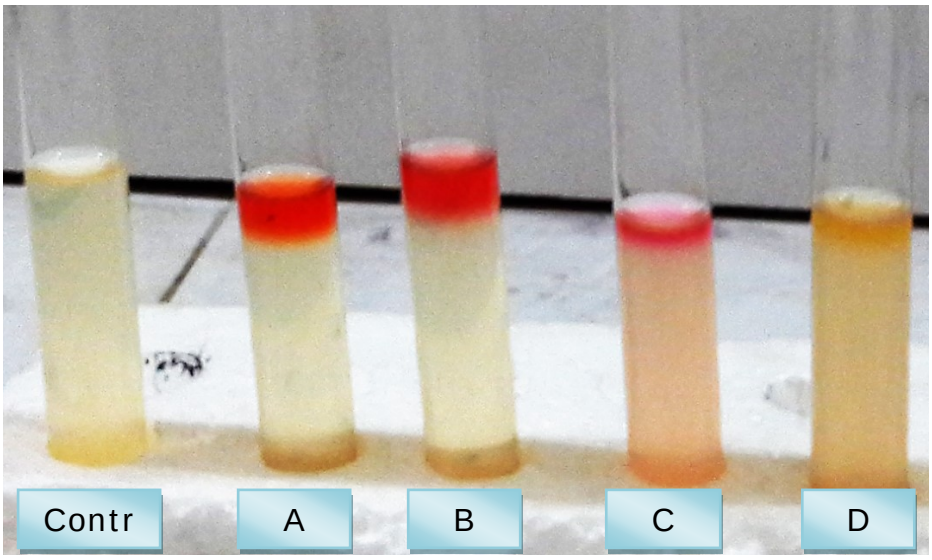


Plate 18: MR test results (Right) A= *E. coli* (Positive), B= *Staphylococcus* (Positive), C= *Salmonella* (Positive*)*, D= *Klebsiella* (Negative) and uninoculated control (Left).

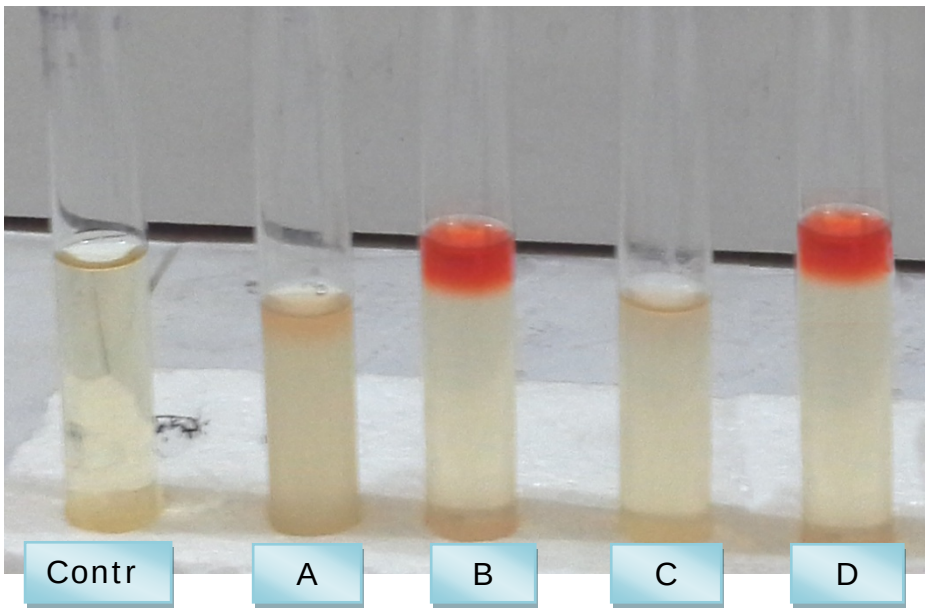


Plate 19: VP test results (Right) A= *E. coli* (Negative), B= *Staphylococcus* (Positive), C= *Salmonella* (Negative*)*, D= *Klebsiella* (Positive) and uninoculated control (Left).

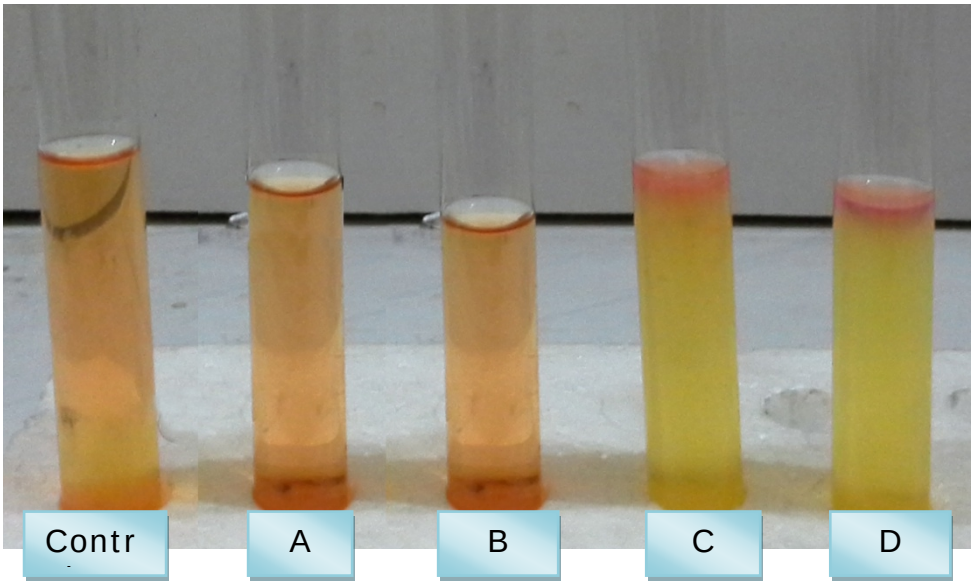


Plate 20: MIU test results (Right) A= *Klebsiella* (Negative), B= *Staphhylococcus* (Negative), C= *Salmonella* (Positive), D= *E. coli* (Positive) and uninoculated control (Left).

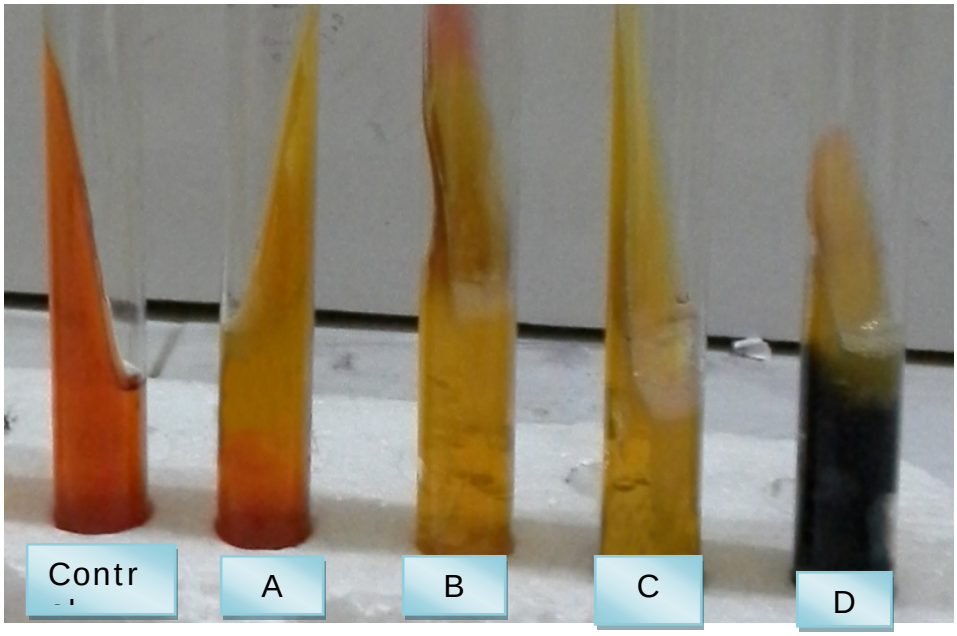


Plate 21: TSI test results (Right) A= *Staphylococcus* (S=A, B=A, Gas= -, H_2S= -), B= *Klebsiella* (S=A, B=A, Gas= +, H_2S= -), C= *E. coli* (S=A, B=A, Gas = +, H_2S= -), D= *Salmonella* (S=Al, B=A, Gas= +, H_2S= +) and uninoculated control (Left).

Legend: (S= Slant, B= Butt, A= Acid, B= Alkaline, (+) = Positive, (-) = Negative)

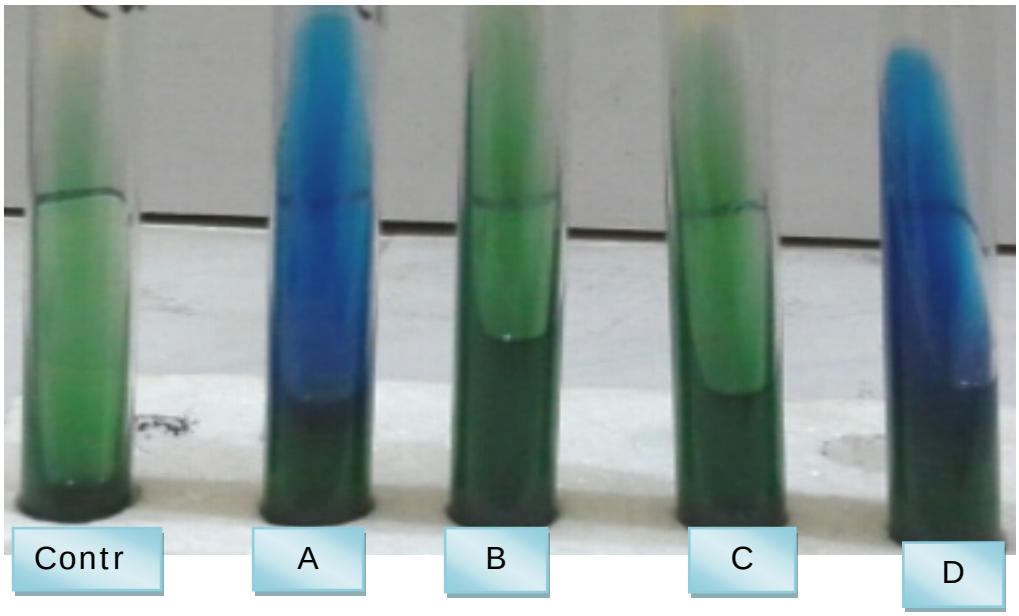


Plate 22: Citrate utilization test results, (Right) A= *Klebsiella* (Positive), B= *E. coli* (Negative), C= *Salmonella* (Negative), D= *Staphylococcus* (Positive), and uninoculated control (L[illegible])

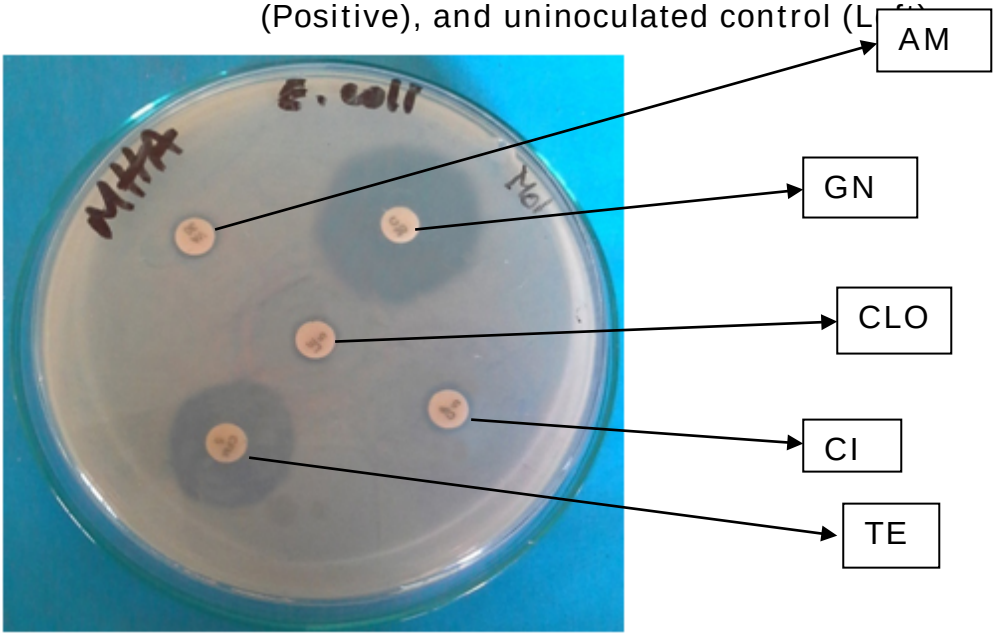


Plate 23: Results of antibiotic sensitivity test of *E. coli* (AMP= Ampicillin, GN= Gentamycin, CLO= Cloxacillin, CIP= Ciprofloxacin, TE= Tetracycline).

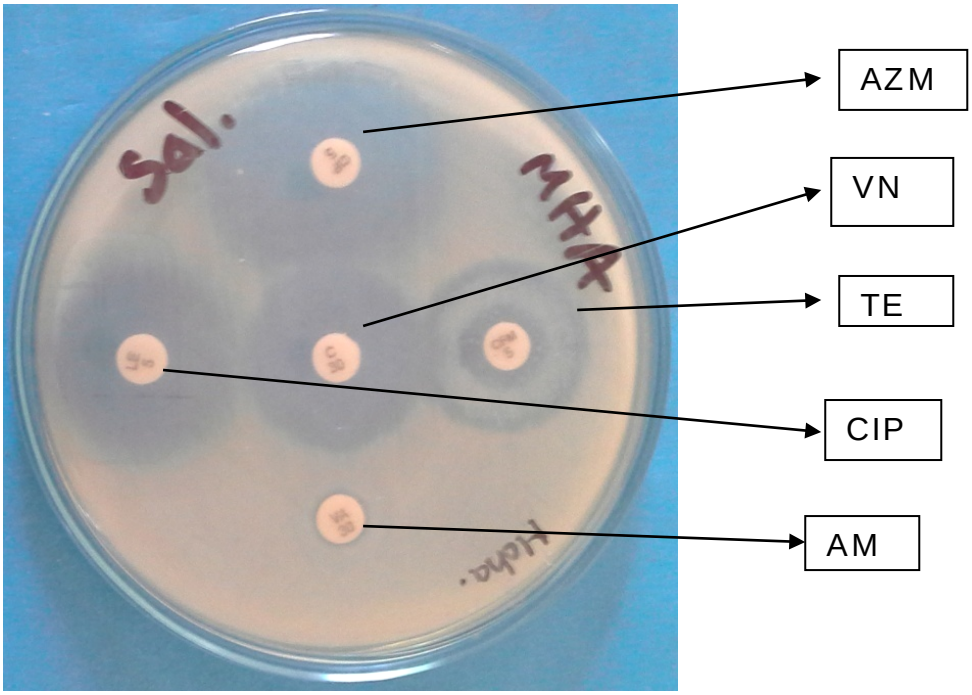


Plate 24: Results of antibiotic sensitivity test of *Salmonella* spp. (AZM= Azithromycin, VN= Vancomycin, TE= Tetracycline, CIP= Ciprofloxacin, AMP= Ampicillin)

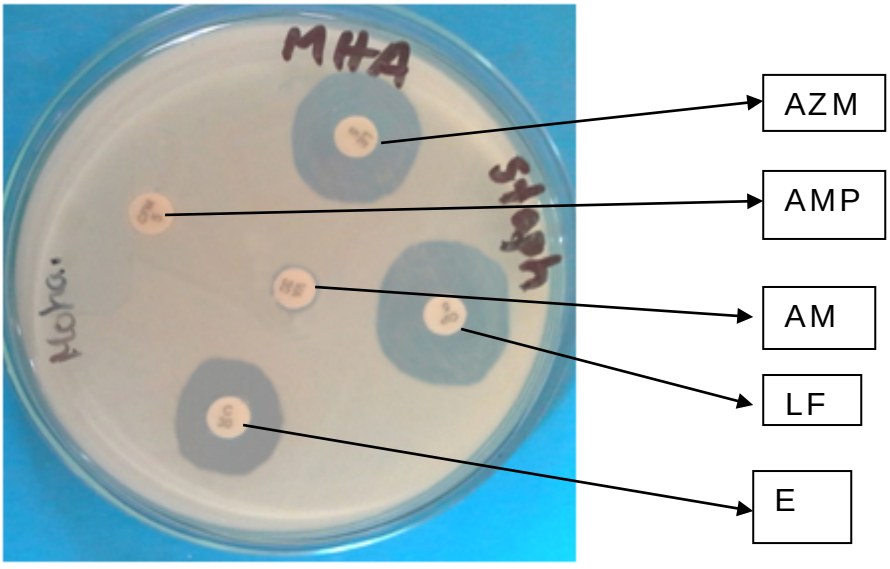


Plate 25: Results of antibiotic sensitivity test of *Staphylococcus* spp. (AZM= Azithromycin, AMP= Ampicillin, AMX= Amoxycillin, LF= Levofloxacin, E= Erythromycin)

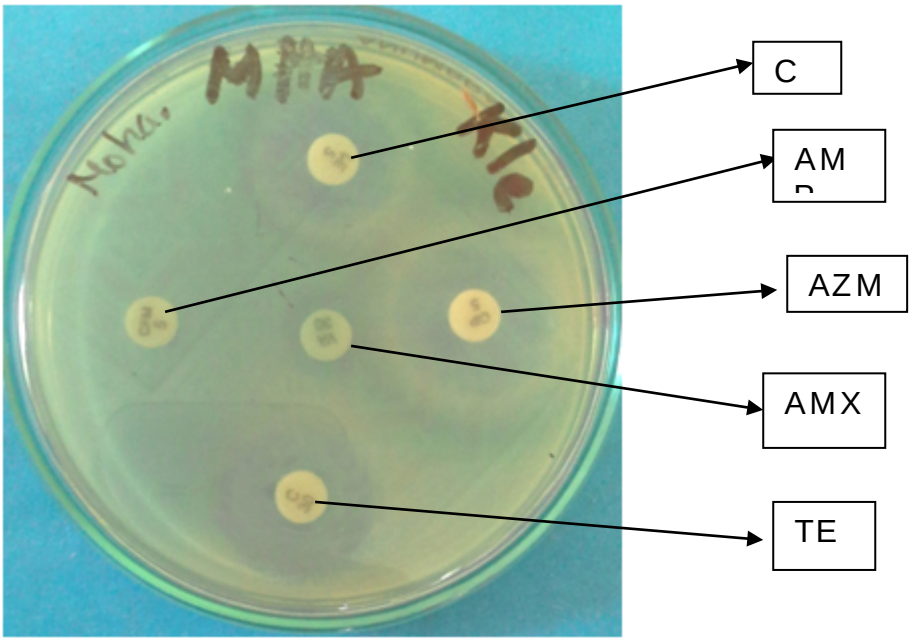


Plate 26: Results of antibiotic sensitivity test of *Klebsiella* spp. (C= Chloramphenicol, AMP= Ampicillin, AZM= Azithromycin, AMX= Amoxicillin, TE= Tetracycline)

CHAPTER 5

DISCUSSION

Fruit juices are very popular among the people of all ages around the world. Also in Bangladesh, fresh fruit juice is becoming more and more popular as they are usually tastier than soft drinks. Advantage of fresh fruit juices is that they are comparatively available in any local market or road side. Fruit juices by their very nature contain various organisms. Clearly, many of these microorganisms may be harmful for health. By detecting bacterial load in the juice, it apparently gives an idea about the quality of the sample. Therefore, total viable count (TVC), total coliform count (TCC) and total Staphylococcal count (TSC) of some available fresh fruit juices were carried out in the present experiment.

Bacterial load of different freshly prepared fruit juices were shown in the table 2. From the results it is clear that all the juices contain a significance amount of microorganisms. Most of the fruit juice samples contained higher load of microbes than the Gulf Standard (Gulf Standards, 2000) for foods described in table 4. The highest bacterial load 5.6×10^{7} cfu/ml of fresh fruit juice sample was found in a sugarcane juice (sample, S-15), collected from Gopalgonj bazar, Dinajpur and the lowest load was 1.8×10^{4} cfu/ml found in an apple juice (sample, S-20) collected from road side at HSTU campus. Variations in TVC in different fruit juices may be due to the unhygienic maintenance during preparing the juice. Rahman et al. (2011) reported that the highest total viable bacterial count 2.4×10^{4} cfu/ml in fresh fruit juice which was found to be lower than this study. Tasnim et al. (2010) also found the load of viable bacteria in processed juice samples within the standard limit in the average of 10^{3} cfu/ml. Bagde and Tumane (2011) found that total bacterial counts in juice samples ranged between 2.0 $x10^{6}$ to 1.0 $x10^{5}$ cfu/ml in Nagpur, India. Microorganisms can enter fruits and vegetables through damaged surfaces, such as punctures, wounds, cuts and breaks that occur during growing or harvesting (Durgesh et al., 2008). Contamination from raw materials and equipment, additional processing conditions, improper handling, prevalence of unhygienic conditions

contribute substantially to the entry of bacterial pathogens in juices prepared from these fruits or vegetables (Oliveira et al., 2006; Nicolas et al., 2007; Durgesh et al., 2008; Odu and Adeniji, 2013).

Most of the fruit juices in our study were found to be unfavorable for consumption because many of them showed the presence of coliforms (*E. coli* and *Klebsiella* spp.). The presence of coliform in fruit juice is not allowed by safe food consumption standard (Andres et al., 2004). The highest total coliform count was found 5.76×10^6 cfu/ml in a wood apple fruit juice (sample, S-29) collected from Nimnagar at Dinajpur town, and the lowest coliform count was found 1.36×10^3/ml in an orange juice (sample, S-9), collected from Modern more at Dinajpur town. In Bangladesh, M. Shakir Uddin Ahmed et al. (2009) showed the presence of *E. coli* ranging from 43 to >2400/100 ml in different types of vended squeezed fruit juices in Dhaka city. In India, the fruit juices were heavily contaminated by *E. coli* (Durgesh et al. 2008) (Tambekar et al. 2009). In comparison with these studies, large numbers of coliforms were found in this study, which we can see from table 2 and all of the sample were found to exceed the Gulf standards (Gulf standards, 2009).

Coagulase-positive Staphylococci may cause human disease through the production of toxins. Effective levels of toxin formation require a high number of microorganisms (approximately 10^5-10^6 micro-organisms per ml of food) (IDF, 1994). A few reports have shown the prevalence of staphylococci in fruit juice samples (M. Shakir Uddin Ahmed et al., 2009; Tambekar et al., 2009). In our study, staphylococci were found in 30 out of 40 tested samples. The highest total Staphylococcal count of fresh fruit juice sample 5.85×10^6 cfu/ml was found in a sugarcane juice (sample, S-16), collected from Bahadur bazar at Dinajpur town (Table-2). The entry of *Staphylococcus aureus* in juices may be attributed to contact with the outer surface of fruits during juicing, survival and growth of foodborne pathogens on surfaces of fruits and vegetables have been demonstrated (Banwart, 1989).

In fruit samples there were no report about *Salmonella* prevalence in fruit juice samples. In this study, some *Salmonella* spp. was found in some tested samples but the number was very low. From Table 2 it can be found that faecal contamination and the concomitant presence of *Salmonella* 12.5% (5 out of 40 samples) was a cause of concern: it is possible that *Salmonella* may have gained entry through water because vendors do not use boiled water and they may use tap water which may be contaminated and this water is commonly used for diluting juices or other ingredients and utensils used for washing and preparing juices, alternately, the possibility of contamination of fruits through improperly treated irrigation water cannot be ruled out; survival and entry of enteropathogens including *Salmonella* have been shown in crops, irrigated with contaminated sewage (Beuchat, 1998).

Rashed et al. (2012) used a new aspect on their investigation comparative to the previous related ones is the study of antibiogram of the pathogenic isolates found in the juice samples. They found that *E. coli* isolates highly resistant against ciprofloxacin (61%), nalidixic acid (71%) and ceftriaxone (57%). *Klebsiella* spp. showed higher resistance against ampicillin (74%), ciprofloxacin (86%), piperaciline (88%), amoxicillin (72%), ceftriaxone (97%) and nalidixic acid (61%). *Staphylococcus* spp. showed resistance against ampicillin (93%), piperaciline (75%), amoxicillin (92%) and vancomycin (63%).

In this study, *E. coli* showed resistance against Ampicillin (100%), Ciprofloxacin (58%), Cloxacillin (89%), and sensitive to Gentamycin (95%), Tetracycline (90%) (Table-10). *Salmonella* spp. showed resistance against Ampicillin (100%), Vancomycin (20%) and sensitive to Ciprofloxacin (100%), Azithromycin (100%), Vancomycin (80%) and Tetracycline (60%) (Table-11). *Staphylococcus* spp. showed resistance against Ampicillin (87%), Amoxicillin (93%) and sensitive to Azithromycin (87%), Levofloxacin (93%) and Erythromycin (80%) (Table-12). *Klebsiella* spp. showed resistance against Ampicillin (100%), Amoxicillin (75%) and sensitive to Chloramphenicol (100%), Tetracycline (75%) and Azithroycin (100%) (Table-13). Such drug resistance properties may render these

pathogens cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics.

The present study has been carried out to investigate the bacteriological quality of fresh fruit juice collected from different areas around Dinajpur city. Where, in the study exhibited the presence of *E. coli, Salmonella* spp., *Staphylococcus* spp. and *Klebsiella* spp. in fresh fruit juice sample. The total viable counts (TVC), total coliform counts (TCC) and total Staphylococcal counts (TSC) of the fresh fruit juice samples were above the normal limit. These high counts indicate heavy bacterial contamination of fruit juice during handling since they are liquid, which could have contributed to the development as well as multiplication of these contaminants. Also, contamination can occur within fruits and materials used for the production of the juice as well as poor sanitation, extraction, raw material contaminations (often from insect damage), lack of both proper heat sterilization and adequate quality control during processing of fruit juice. The study has also shown that these fresh fruit juices are not sterile and thus can favour the growth of microorganisms when conditions become favourable, which could pose a public health risk to their consumers.

CHAPTER 6

SUMMARY

The present study was conducted for the bacteriological analysis with antibiotic resistance pattern of bacteria isolated from fresh fruit juice samples. Samples were collected during the period of January to June 2017, from different areas around Dinajpur city of Bangladesh. The laboratory works were conducted in the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur. A total of forty (40) fresh fruit juice samples from different areas around Dinajpur city were collected for this study. Standard plate count techniques were followed to assess Total viable bacterial count (TVC), Total coliform count (TCC), and Total Staphylococcal count (TSC). A series of bacteriological methods were used for the isolation and identification of different types of bacteria and to determine the antibiotic resistance pattern of those isolates to different antibiotics. Different types of ordinary, enriched and selective media such as Nutrient broth, Nutrient agar, Mannitol Salt agar, Eosine Methylene agar, MacConkey Agar and Mueller Hinton agar were used for this study. Biochemical properties of the isolated bacteria were studied by indole test, MR-VP test, MIU test, TSI test and Citrate utilization test. On the basis of morphology, staining, cultural and biochemical characteristics, the isolated organisms were identified as, *Escherichia coli, Salmonella* spp., *Staphylococcus* spp., *and Klebsiella* spp. Bacteriological examination were done of about total 40 samples. Out of 40 samples, 38 were positive for *Escherichia coli* (95%), 5 were positive for *Salmonella* spp. (12.5%), 30 were positive for *Staphylococcus* spp. (75%), and 4 were *Klebsiella* spp. (10%).

The antibiotic study revealed that the isolates of *Escherichia coli* were resistant to Ampicillin (100%), Ciprofloxacin (58%), Cloxacillin (89%) and sensitive to Gentamycin (95%), Tetracycline (90%). The isolates of *Salmonella* spp. were resistant to Ampicillin (100%), Vancomycin (20%) and sensitive to Ciprofloxacin (100%), Azithromycin (100%), Vancomycin (80%) and Tetracycline (60%).

The isolates *staphylococcus* spp. of were resistant to Ampicillin (87%), Amoxicillin (93%) and sensitive to Azithromycin (87%), Levofloxacin (93%) and Erythromycin (80%. The isolates *Klebsiella* spp. were resistant to Ampicillin (100%), Amoxicillin (75%) and sensitive to Chloramphenicol (100%), Tetracycline (75%) and Azithroycin (100%). Such drug resistance properties may render these pathogens cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics.

CHAPTER 7

CONCLUSION

From the data presented in the current study, it can be concluded that the bacteriological quality of most of the fresh fruit juice samples collected from different areas around Dinajpur city were not satisfactory as *E. coli, Salmonella* spp., *Staphylococcus* spp. *and Klebsiella* spp. were detected from the samples. The isolated bacteria were found resistant to different antibiotics such as Ampicillin, Amoxicillin, Ciprofloxacin, Cloxacillin, Vancomycin etc. The lack of knowledge on safe fruit juice preparation as well as the contamination sources can contribute to the elevation of pathogens in prepared juices. It is therefore, essential for the people who handle and prepare juices, to be properly trained on safe fruit handling technique. Regular monitoring of the quality of fruit juices for human consumption is recommended to avoid any future bacterial pathogen outbreak.

The practice of consuming fresh fruit juices cannot be stopped on nutritional grounds nor the street vendors prohibited from selling such items since such activities provide them with a source of livelihood, government agencies such as Bangladesh Council of Scientific and Industrial Research (BCSIR) and Bangladesh Standard and Testing Institution (BSTI) must adopt measures to educate the vendors about food safety and hygienic practices and enforce adequate guidelines for juices especially freshly prepared juices: such norms and conventions, currently do not exist in Bangladesh.

In the context of this study, it may be concluded that,

1. The presence of *E.coli*, *Salmonella* spp.*, Staphylococcus* spp. and *Klebsiella* spp. in most of the samples are of public health concern.
2. Total viable count (TVC), Total coliform count (TCC) and Total staphylococcal count (TSC) were successfully performed from different fresh fruit juice samples.
3. High bacterial counts in all fruit juice samples indicate that consumption of these fresh fruit juice is harmful for public health.

4. The drug resistance properties of isolated bacteria can cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics.

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APPENDICES

APPENDIX 1

Composition of Different Media

1. Nutrient agar (Hi Media)

Ingredients:

g/L
Peptic digest of animal tissue 5.0
Sodium chloride 5.0
Beef extract 1.5
Yeast extract 1.5
Final pH (at 25^{0}C) 7.4 ± 0.2

2. Eosine methylene blue Agar (Hi Media)

Ingredients:

g/L
Peptic digest of animal tissue 10
Lactose 5.0
Sucrose 5.0
Dipotassium phosphate 2.0
Eosin - Y 0.40
Methylene blue 0.065
Agar 20.0
Final pH (at 25^{0}C) 7.2 ± 0.2

3. MacConkey agar (Hi-media)

Ingredients:
g/L
Peptic digest of animal tissue
17.0
Protease peptone
3.0
Lactose monohydrate
10
Bile salt
1.5
Sodium chloride
5.0
Agar-agar
15.0
Neutral red
0.03
Final pH (at 25^{0}C)
7.1 ± 0.2

4. Mannitol Salt Agar

Component
Amount (g/L)
Proteose peptone
10.0
Beef extract
1.0
Sodium chloride
75.0
D-mannitol
10.0
Phenol red
0.025
Agar
15.0
Final pH 7.4 ± 0.2 at
25°C

5. Simmon's Citrate Agar

Component
Amount (g/L)
Magnesium sulphate
0.2
Ammoniun dihydrogen phosphate
1.0
Dipotassium phosphate
1.0
Sodium citrate
2.0
Sodium chloride
5.0
Bacto agar
15.0
Bacto bromo thymol blue
0.08

6. Mueller Hinton Agar

Component
Amount (g/L)
Beef infusion
300.000
Casein acid hydrolysate
17.500
Starch
1.500
Agar
17.000
Final pH(at 25°C)
7.3±0.1

7. TSI agar (Hi Media)

Ingredients:
g/L
Peptic digest of animal tissue
10.00
Casein enzymic hydrolysate
10.00
Yeast extract
3.00
Beef extract
3.00
Lactose
10.00

Sucrose
10.00
Dextrose
1.00
Sodium chloride
5.00
Ferrous sulphate
0.20
Sodium thiosulphate
0.30
Phenol red
0.024
Agar
12.00
Final pH(at 25°C)
7.4 ± 0.2

8. MIU medium base (Hi Media)

Ingredients:
g/L
Casein enzymic hydrolysate
10.00
Dextrose
1.00
Sodium chloride
5.00
Phenol Red
0.01
Agar
2.00
Final pH(at 25°C)
6.8 ± 0.2

9. MR-VP medium (Hi Media)

Ingredients:
g/L
Buffered peptone
7.00
Dextrose
5.00
Dipotassium phosphate
5.00
Final pH (at 25°C)
6.9 ± 0.2

10. Sugar media

Ingredients:

g/L
a. Peptone water
Bacto-peptone
10.0 gm
Sodium chloride
5.00 gm
0.5% phenol red
0. 10 ml
Distilled water
1000 ml

b. Sugar solutions
Individul sugar
5.00 gm
Distilled water
100 ml

c. Sugar media preparation
Pepton water
4.50 ml
Sugar solution
0.50 ml

11. Peptone water

Ingredients:
g/L
Peptone
1.00 gm
Distilled water
1000 ml

APPENDIX 2

Preparation of reagents

1. Kovacs reagent

P-dimethyl aminobenzal dehyde 5 gm
Amylalcoho
l75 gm
Conc.HCL
25 ml

2. V-P reagent 1

5% alpha –naptholin absolute ethyl alcohol

3. V-P reagent 2

40%potassium hydroxide containing 0.3 creatine. The ingredients were dissolved by heating gently over steam bath. When in solution add 0.05gm of cotton blue dye.

4. Phosphate buffered solution

Sodium chloride
8 gm
Disodium hydrogen phosphate
2.8 gm
Potassium chloride
0.2 gm
Potassium hydrogen phosphate
0.2 gm
Distilled water to make
1000 ml

5. Methyl red solution

Methyl red
0.05 gm
Ethanol (absolute)
28 ml
Distilled water
22 ml

6. Phenol red solution

0.2% aqueous solution of phenol red

7. Potassium hydroxide solution

40% aqueous solution of KOH

8. Gram stain solution

 Stock crystal violet

Crystal violet
10 gm
Ethyl alcohol (95%)
1000 ml

 Stock oxalate solution

Ammonium oxalate
1 gm
Distilled water
1000 ml

 Lugols iodine solution

Iodine crystal
1 gm
potassium iodide
2 gm

 Ethyl alcohol
250 ml

 Acetone
250 ml

 Counterstain

Safranine
2.5 ml
Ethyl alcohol (95%)
100 ml

Safranine working solution
The stock safranine is diluted 1:4 with distilled water.